RESEARCH ARTICLE

Historical evidence for context-dependent assessment of *Erigeron canadensis* invasions in an 18th-century European landscape

Ingo Kowarik[1]

1 *Technische Universität Berlin, Institute of Ecology, Rothenburgstr. 12, D-12165 Berlin, Germany*

Corresponding author: Ingo Kowarik (kowarik@tu-berlin.de)

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Abstract

Understanding the historical roots of invasion science provides insights into early perceptions of invasive species, allows us to trace the evolution of the discipline over time, and helps contextualize modern research. This paper analyzes work by Christian Ludwig Krause, published 250 years ago, on the invasion of an 18th-century European landscape by *Erigeron* [*Conyza*] *canadensis* (Canadian horseweed), one of the most common invasive species today and a widespread agricultural weed. Here an analysis is conducted of the ecological consequences and underlying mechanisms Krause described, how he evaluated *E. canadensis* invasions in different land-use systems and how his insights align with existing knowledge. Krause identified copious seed production and long-distance dispersal by wind as key mechanisms for the formation of dominant stands on degraded sandy soils. He recognized various ecosystem services associated with population establishment, such as erosion control, increased soil fertility, and the facilitation of other species. While Krause highlighted the benefits of *E. canadensis* invasions for the recovery of degraded grasslands and fields, he also acknowledged this introduced species as a troublesome weed in gardens. Thus, Krause's work is not only an early report on the invasion of a cultural landscape subject to wind erosion but also an early example of a context-dependent invasion assessment, illustrating both positive and negative impacts of the same species in different environments. Krause's perspective may encourage current assessments of *E. canadensis* not solely based on its presence or frequency, but on documented ecological and socioeconomic effects and their associated benefits or harms. As Krause impressively demonstrated 250 years ago, these effects can differ starkly in different environments, necessitating multiple responses to the same species.

Introduction

The establishment of invasion science as a discipline represents a remarkable achievement of the 20th century, and as the spread of non-native species accelerates, with associated challenges to biodiversity conservation, health, and economic sectors (Pyšek et al. 2020a; Zenni et al. 2021), the importance of the discipline grows. While the international SCOPE program on the ecology of biological invasions, which began in 1982, is often considered the foundation of modern invasion science (Simberloff 2011), the discipline has many historical roots. Elton's book from 1958 is a milestone (Richardson and Pyšek 2008), but invasion science has much deeper roots in Europe and elsewhere (e.g. van Wilgen 2020). In "The Origin of Species", for example, Darwin (1859) describes several invasion mechanisms (Ludsin and Wolfe 2001). Many other historical sources remain hidden, especially if they were not written in English. Acknowledging these roots allows us to better trace the evolution of the discipline over time and helps contextualize modern research.

Indeed, there are early works that describe some stages of the invasion process (as defined by Richardson et al. 2000), particularly species introduction, spread and naturalization. Since the Middle Ages, herbal books, garden directories, horticultural and forestry works have documented the influx of introduced species (e.g., Wein 1914). Starting at the end of the 17th century, regional floras and related works list species' spontaneous occurrences, providing documentation of their spread (e.g., de Tournefort 1698). In the 19th century, Watson (1847) and de Candolle (1855) developed initial frameworks, distinguishing native vs introduced plants and also addressing species' naturalization. Based on this, the Swiss botanist Thellung (1905, 1918/19) developed a comprehensive classification system that described species' introduction pathways and invasion success and applied it to regional floras. His "Flore adventive de Montpellier" (Thellung 1912) is a largely neglected milestone of bioecologically based invasion research, providing first quantifications for species' naturalization and the strength of introduction pathways (Kowarik and Pyšek 2012).

In ecological classifications (Richardson et al. 2000), species that have reached the fourth stage of the invasion processes by spreading beyond their point of introduction are considered "invasive." Many early floristic works include species abundance data that indicate advanced invasion success by this definition. However, distribution maps documenting species's spread for larger areas were not produced until the second part of the 20th century as Pyšek and Prach (2003) show for the Czech Republic. In contrast, the IUCN and other approaches in environmental policies classify species as invasive when these induce negative impacts on biodiversity and/or effect socioeconomic damage (Pyšek et al. 2020a). Such negative invasion impacts had been already addressed in the 19th century. Darwin (1859: 380), e.g., mentioned profound changes to the biota of oceanic islands such as a decrease in native species, driven by naturalized species, as "the first stage towards extinction". The German botanist von Chamisso (1827: 49) perceptively addressed invasion processes and related consequences about 30 years earlier:

"Where the civilized man settles, the view of nature changes … His plantings and seeds spread around his dwelling … In his gardens and fields, among the plants he cultivates, a multitude of other plants grow as weeds … Where he has not taken all the space, the plants that were dependent on him move away from him, and even the wilderness, which his foot has not yet touched, changes its form."
[translation of all citations in German by IK]

Potential benefits of introduced species, beyond cultivated species, were considered only in the last decades in cost/benefit analyses (e.g., U.S. Congress 1993) and were later included in impact assessment schemes, highlighting the significant relationship between impact assessment and societal values (Bartz et al. 2010; Jeschke et al. 2014). Schlaepfer et al. (2012) emphasized the often underestimated conservation benefits of non-native species, and Dickie et al. (2014) illustrated the relevance of non-native species to socioeconomic sectors. Another recent topic of study is the context-dependence of invasion impacts. These impacts differ across biogeographic and ecological contexts. However, whether a change due to introduced species is regarded as a benefit or damage (or as a neutral effect) is also a question of whether it supports or conflicts with a particular set of values, which often differ within and between societies (Bartz et al. 2010). Context-specific assessments of plant invasions represent a challenge in invasion science (Pyšek et al. 2020b) and need to bridge ecological and societal realms (Sax et al. 2022).

Our ways of assessing invasion impacts in different contexts also likely have an older, yet largely hidden history. As a step towards illuminating these roots of invasion science, this paper analyzes an 18ᵗʰ-century example, included in a book by Christian Ludwig Krause (1706–1773) published 250 years ago (Krause 1773). Herein, Krause described the spread of the North American annual *Erigeron canadensis* L. (syn. *Conyza canadensis* (L.) Cronquist, Canadian horseweed) in a Central European landscape. He presented mechanisms of spread and associated ecological consequences, and he assessed the latter in terms of benefits and harm.

This is an intriguing case as *E. canadensis* is now the most widely spread non-native species in Europe (Lambdon et al. 2008) and beyond (e.g. Xu et al. 2012), colonizing a broad range of anthropic and (near)-natural ecosystems such as arable fields (Zimmermann et al. 2015), old fields (Prieur-Richard et al. 2000; Liendo et al. 2021), post-industrial sites (Zaplata et al. 2011; Anibaba et al. 2023), urban habitats (Dyderski and Jagodziński 2016), grasslands (Axmanová et al. 2021), coastal dunes (Giulio et al. 2020), floodplains (Anđelković et al. 2022), and other open sites, often with species-poor communities (Padullés Cubino et al. 2022). Due to its rapid spread, *E. canadensis* is often described as invasive and can create an economic burden in agricultural systems (Bajwa et al. 2016).

Here, the historical background of 18ᵗʰ-century Brandenburg, now part of Germany, is outlined first, including major environmental challenges of the time. Then Christian Ludwig Krause is briefly introduced together with his connection to introduced species. The subsequent analysis of the *Erigeron* case study addresses these questions: (1) What mechanisms and (2) what ecological consequences of spreading *E. canadensis*

did Krause describe, and to what extent does current knowledge support his insights? (3) How did he address invasion impacts in terms of benefits and harms and can this case be understood as an early precursor of context-dependent invasion assessments?

Historical background

18th-century Brandenburg

Eighteenth-century Brandenburg, today part of Germany, belonged to the Kingdom of Prussia, with Berlin as capital. Prussia's increasing political and economic importance fueled heavy demand for wood as the main building material and energy source. Many forests were converted to agricultural land to nourish the quickly growing population (Hasel and Schwartz 2006). In consequence, only about a quarter of Brandenburg's surface (24.3%) remained covered by forest by the end of the 18th century (Bratring 1804, p. 13). As elsewhere in Europe (McGrath et al. 2015), many of these forest remnants were degraded by manifold uses, resulting in their fragmentation and conversion into grasslands, heaths and fields (Krausch 1968). Over-grazing stimulated wind erosion on exposed sandy sites, rendering adjacent fields unusable due to flying sand. Around 1782, 23 open sand areas, including shifting dunes, each larger than 26 hectares, were documented around Berlin (von Klöden 1832). A major environmental challenge was thus to stabilize the open sand plains and restore agricultural land use and forests.

Christian Ludwig Krause

Christian Ludwig Krause (1706–1773) was renowned among his contemporaries as an influential gardener and owner of a commercial nursery and seed trade in Berlin, which was associated with a highly diverse garden (Kowarik 2023a). A plant directory by Roloff (1746) shows that Krause's garden harbored 2,420 taxa. The garden had been addressed as a privately-owned botanical garden and had more taxa than some other German botanical gardens at the time (Kowarik 2023a). Krause was considered one of the most important German gardeners in the 18th century (Teichert 1865), and his garden was particularly famous as a hub of cultivation and for distribution of newly introduced species (Nicolai 1779). Krause was the first in Berlin to cultivate several introduced species, including *Acer monspessulanum*, which has started to spread only recently (Kowarik 2023b), and others that spread earlier such as *A. saccharinum*, *Catalpa bignonioides*, *Gleditsia triacanthos*, *Myrica cerifera*, and *Pinus strobus*. Krause distributed plants and seeds across Germany and neighboring countries and participated in a supra-regional network of natural history research, centered around Carl von Linné with whom he corresponded for more than 20 years (Dietz 2010; Kowarik 2023a).

Krause published his main work, a 782-page book with horticulture as the focus (Krause 1773), 250 years ago. But the monumental work goes beyond horticulture, with some chapters addressing solutions for pressing environmental challenges of the

time (Kowarik in press): Krause described ways to restore oak woodlands, establish silvicultural pine plantations and hedgerows, and recover degraded land. In one chapter, he reported the natural revegetation of open sand fields by *Erigeron canadensis*, using the name "*Virga aurea, Virginiana annua*" (sensu de Tournefort 1698: 173)—an early report on biological invasions in a rural context.

Methodological approach

The chapter that reports on the *E. canadensis* case (Krause 1773: 405–409) is first evaluated here in terms of the included information on the occurrence of the species, mechanisms of spread, and associated ecological consequences, and how Krause related the latter to benefits or harms. The historical evidence is then contrasted with the current state of knowledge, based on a literature search in the Web of Science and on Wein's (1932) historical study of the introduction and spread of *E. canadensis* in the 17th and 18th centuries.

Results and discussion

Krause's report on *E. canadensis* invasion and related consequences

Krause wrote that the annual species was "brought to us about a hundred years ago". He was aware of the introduced status of *E. canadensis* and its North American origin since the species' name included a reference to North America, specifically Virginia, and the synonymous name mentioned Canada ("Aster *Canadensis annuus*, flore pappose", p. 407). He said that the quantity of seeds produced would "surpass all other species to [his] knowledge" (p. 407) and precisely described the morphological adaptation of "seeds" [achenes] for wind dispersal, which allows them to be "lifted by the air and carried away and borne by wind and storms over many miles" (p. 406). Krause reported highly abundant populations on degraded sandy areas in Brandenburg. He himself "encountered many thousands of plants in certain areas on sandy plains, where they have grown up to three feet [approximately 1 m] high and formed small shrubberies without having been sown" (p. 407).

Krause described benefits associated with *E. canadensis* invasions in sandy areas and illustrated underlying ecological mechanisms related to erosion control, soil formation and the facilitation of subsequent species (p. 407f.). While the species may not be suitable as a fodder plant, he said, "it has its true usefulness in sandy areas where it seeds itself." After it "has emerged in the spring, the wind has no power to pick up the sand and drive such towards good fields; instead, the growth of these plants creates firm and cohesive soil." "As soon as the plants have produced stems, leaves and other light nutritious bodies carried by the wind are deposited among them. These, together with the entire plant that dies in autumn, are dissolved by winter moisture, rain, and snow, and serve as nourishment for other plants, also brought by the wind, which then grow and find sustenance on the sand plains."

Finally, Krause contrasted the benefits of colonizing sand plains with the disservices of *E. canadensis* in gardens (p. 408): "Although this contemptible weed in gardens is of no use due to its astonishing proliferation, only causing much work with weeding and uprooting, it has its true value on light sandy fields."

Krause's insights in light of current knowledge

Introduction history and spread

Krause correctly identified the time of *E. canadensis*'s introduction ("about a hundred years ago", p. 407). Introduced from French colonial territories in North America, *E. canadensis* had probably initially been cultivated in French gardens (Wein 1932). However, the first record was in 1646 from the botanical garden in Altdorf near Nuremberg, Germany (Wein 1932). The species was also an early garden plant in Brandenburg (Gleditsch 1737), growing in Krause's garden in Berlin as well (Roloff 1746, p. 173). Wein (1932) explains why *E. canadensis* was cultivated as a garden plant from the mid-17th century into the 18th century, despite its unremarkable appearance. At that time, there was a fervor for all things French, so the species was positively regarded due to its origin from French colonies and its further distribution through France.

Krause's invasion report about *E. canadensis* is not the first. As early as 1659, its spontaneous spread was documented in the surroundings of Paris. It was described as "la plus commune de la campagne" [the most frequent of the countryside] by the end of the 17th century (de Tournefort 1698: 542). *Erigeron canadensis* was recognized as having escaped from cultivation in other European countries as early as the beginning of the 18th century, including in Brandenburg (since 1710; Wein 1932). Willdenow (1787: 270) described it in his Flora of Berlin as very common in gardens, cultivated fields, disturbed sites, and forests. While the spread of *E. canadensis* was thus previously known, Krause's report is probably the first one to mention abundant dominant populations in sandy areas and to describe associated ecological mechanisms and consequences.

Seed production and dispersal

The significance of copious seed production and long-distance dispersal by wind for the rapid spread of the species was recognized early by French botanists (de Tournefort 1698: 174). Recent studies support the high seed production of *E. canadensis*, which Krause described as surpassing all known species. A 1.5 m tall plant can produce nearly 230,000 seeds, and even a 40 cm tall plant can produce 2,000 seeds (Weaver 2001). In addition, European plants set more seeds, grow taller, and suffer less from co-migrated specialist enemies compared to American plants (Abhilasha and Joshi 2009).

The wind dispersal reported by Krause over "many miles" is also supported by current studies. Seed trap experiments revealed that while 99% of seeds fell within 100 m, some were moved at least 500 m (Dauer et al. 2007). For seeds lifted 10 m above

the ground, dispersal of up to 36.5 km was modeled under weak wind conditions (1–4 m s⁻¹), and up to 165 km under stronger wind (5–7 m s⁻¹), with 14% of seeds being moved more than 10 km (Liu et al. 2018). Considering that seeds have been detected at heights of 140 m, where they can reach the Planetary Boundary Layer and its wind speeds of over 20 m/s, dispersal of more than 500 km is feasible (Shields et al. 2006). This well explains the rapid spread after initial introduction in gardens and its wide distribution across Europe as already suggested by Wein (1932).

We now recognize that human-mediated seed dispersal plays a role in quickly establishing large dominant populations, for example through seed attachment to shoes or vehicles. Accordingly, Zaplata et al. (2011) explained the sudden occurrence of dense *E. canadensis* stands in post-mining sites by seed influx through trucks. In fact, seeds of this species were the second most commonly transported by traffic in Berlin, surpassed only by rye seeds from transport losses (von der Lippe and Kowarik 2007a, b). The dominant stands described by Krause could, therefore, have resulted from a combination of different dispersal pathways that were not fully understood in the 18ᵗʰ century.

Erosion control and soil improvement

Krause's report on the reduction of wind erosion on open sandy areas owing to dense *E. canadensis* stands appears plausible, and he is likely the only one describing this benefit for agricultural land use. Recent studies confirm the occurrence of *E. canadensis* in various environments with sandy soils across Europe (Zaplata et al. 2011; Zimmermann et al. 2015; Giulio et al. 2020; Axmanová et al. 2021). Although wind erosion in agricultural systems remains a significant and economically relevant problem today (Riksen and De Graaff 2001), erosion control by *E. canadensis* has not been considered in modern times. The wealth of existing literature on agricultural fields mostly focuses on negative consequences, such as yield losses due to competition with crops (Bajwa et al. 2016).

Current studies support the soil improvement highlighted by Krause through the capture of airborne material and the decomposition of its own biomass. Although *E. canadensis* has a lower decomposition rate than other pioneer plants, with a C/N ratio of 13.3 for leaf and 23.3 for litter (Schädler et al. 2003), it likely enhances soil formation and nutrient status on open sandy soils, particularly in the absence of other species. *Erigeron canadensis* invasions correlate with increased nutrient levels (N, P), decreased salinity, and improved soil structure in steppe soils (Zhang et al. 2021). Experiments have demonstrated soil nutrient content and enzymatic activities increasing with the abundance of *E. canadensis* (Zhang et al. 2020).

Facilitation of other species

Krause has described how abundant populations stimulate soil formation and nutrient enrichment on open sandy soils, thereby creating the foundation for the establishment of other species. This corresponds to the successional model of "relay floristics" described by Egler (1954) for old fields, where pioneer species prepare the site for

subsequent species, which then outcompete them. Facilitation of other species, as diagnosed by Krause, is indirectly confirmed by long-term successional studies. On both old fields (Schmidt 1981; Bonet and Pausas 2004; Dölle et al. 2008) and sandy pioneer sites in post-mining landscapes (Zaplata et al. 2013; Schaaf et al. 2013), the dominant *E. canadensis* and other annuals are largely replaced by perennial species after a few years. Therefore, Albert et al. (2014) recommend allowing succession in sandy old fields as a promising pathway to grassland restoration, despite the initial dominance of non-native annuals and given the proximity of target species.

Different abundances of *E. canadensis* might also induce different effects on plant community composition (Wang et al. 2021). A few conflicting experimental studies indicate negative, positive, or neutral effects, related to allelopathic effects (Shaukat et al. 2003; Djurdjević et al. 2011) and changes in soil biota (Řezáčová et al. 2020, 2021, 2022). However, it remains to be clarified whether changes in local communities caused by *E. canadensis* will lead to a long-term decline in species beyond the local level, potentially posing a threat to biodiversity. While the species frequently occurs in dry grassland on sandy soils in Brandenburg, negative effects on species of conservation concern are not expected since it usually temporarily colonizes gaps following disturbance (D. Lauterbach, personal communication, Fig. 1).

Figure 1. Decaying population of *Erigeron canadensis* in a dry grassland site in western Brandenburg (near Gülpe), previously subjected to mechanical disturbance (Photo by Daniel Lauterbach, October 2023).

Conclusions

Examining historical roots of current invasion science provides insights into the early perceptions of invasive species, allows us to trace the evolution of the discipline over time and helps contextualize modern research findings. Early historical roots of invasion science, since the 17th century, mainly focused on the introduction, spread, and naturalization of species. The negative impacts of biological invasions have been addressed only since the 19th century, but potential benefits have not received much attention–if any. Thus, Krause's 18th-century chapter on the colonization of degraded sand areas by *E. canadensis* is more than just an early account of plant invasions in the pre-industrial cultural landscape. It is one of the first known works on the benefits associated with plant invasions, covering a range of regulating ecosystem services such as erosion control, increase in soil fertility, and the revegetation of degraded land (Fig. 2).

Krause refrained from making assessments based solely on the copious abundance of *E. canadensis*, nor did he categorize the species as inherently undesirable or beneficial. Instead, he considered its different effects in various ecosystems, making his work an early example of a context-dependent assessment of plant invasions. He reported *E. canadensis* as a troublesome weed in gardens, which aligns with the current perspective on the species as an agricultural weed (Bajwa et al. 2016). At the same time, he demonstrated the beneficial effects of *E. canadensis* invasion in another land-use system, specifically in the recovery of degraded rural landscapes. Conducting such differentiated, nuanced assessments in different ecological and socioeconomic contexts is challenging in invasion research today (Bartz and Kowarik 2019; Pyšek et al. 2020b) as is the consideration of many unexploited opportunities to consider benefits introduced species can support (Sax et al. 2022).

Threats to biological diversity due to plant invasions were not yet a topic during Krause's time due to the prevailing utilitarian view of nature's benefits (Meyer and
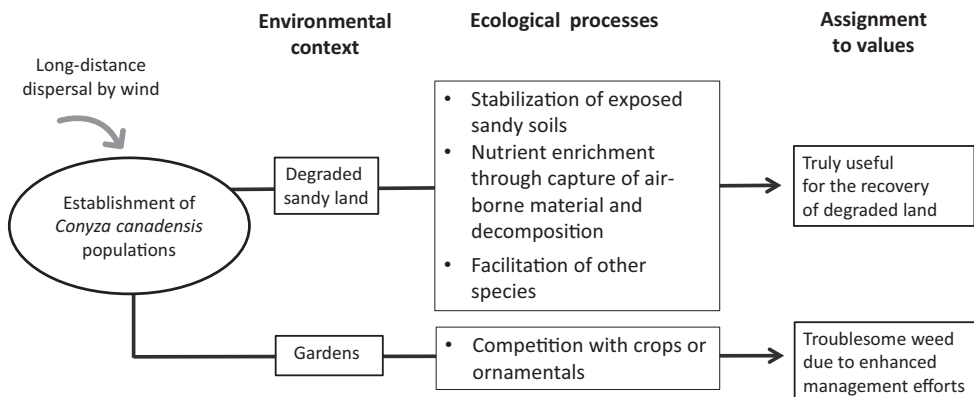

Figure 2. An early 18th-century example of context-dependent assessment of biological invasions: Invasions of different land-use systems by *Erigeron canadensis*, underlying ecological mechanisms, and their evaluation in the work by Krause (1773).

Popplow 2004). Current ecological studies indicate that, at least in the European context, the dominance of *E. canadensis* in sandy and other open habitats is largely caused by disturbance and does not hinder the recovery of species-rich, perennial vegetation. Allowing succession can thus even be a promising pathway for restoration (Albert et al. 2014).

It remains an open question whether the establishment of low-abundance populations of *E. canadensis* across many vegetation types in Europe with possible effects on neighbouring species actually challenges species conservation at the community or landscape levels. The example of Krause's work may encourage assessments of *E. canadensis* not solely based on its occurrence or frequency, but on demonstrated ecological effects and their associated benefits or harms. As Krause impressively demonstrated 250 years ago, these effects can differ starkly in different contexts, arguing for multiple responses to the same species.

Acknowledgements

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RESEARCH ARTICLE

A multi-taxa assessment of aquatic non-indigenous species introduced into Iberian freshwater and transitional waters

Jose M. Zamora-Marín[1,2], Ana Ruiz-Navarro[1,3], Francisco J. Oficialdegui[1,4],
Pedro M. Anastácio[5], Rafael Miranda[6], Pablo García-Murillo[7], Fernando Cobo[8],
Filipe Ribeiro[9], Belinda Gallardo[10], Emili García-Berthou[11], Dani Boix[11],
Leopoldo Medina[12], Felipe Morcillo[13], Javier Oscoz[6], Antonio Guillén[1],
Antonio A. Herrero-Reyes[1], Francisca C. Aguiar[14], David Almeida[15], Andrés Arias[16],
César Ayres[17], Filipe Banha[5], Sandra Barca[8], Idoia Biurrun[18], M. Pilar Cabezas[19],
Sara Calero[20], Juan A. Campos[18], Laura Capdevila-Argüelles[21], César Capinha[22,23],
André Carapeto[24], Frederic Casals[25,26], Paula Chainho[9], Santos Cirujano[27],
Miguel Clavero[28], Jose A. Cuesta[29], Vicente Deltoro[30], João Encarnação[31],
Carlos Fernández-Delgado[32], Javier Franco[33], Antonio J. García-Meseguer[34],
Simone Guareschi[28], Adrián Guerrero-Gómez[1], Virgilio Hermoso[7],
Celia López-Cañizares[1], Joaquín López-Soriano[35], Annie Machordom[36],
Joana Martelo[9], Andrés Mellado-Díaz[20], Juan C. Moreno[37], Rosa Olivo del Amo[1],
J. Carlos Otero[8], Anabel Perdices[36], Quim Pou-Rovira[38], Sergio Quiñonero-Salgado[35],
Argantonio Rodríguez-Merino[7], Macarena Ros[39], Enrique Sánchez-Gullón[40],
Marta I. Sánchez[41], David Sánchez-Fernández[34], Jorge R. Sánchez-González[42],
Oscar Soriano[36], M. Alexandra Teodósio[31], Mar Torralva[1], Rufino Vieira-Lanero[8],
Antonio Zamora-López[1], Francisco J. Oliva-Paterna[1]

1 Department of Zoology and Physical Anthropology, Faculty of Biology, University of Murcia, CEIR Campus Mare Nostrum (CMN), Murcia, Spain **2** Department of Applied Biology, Centro de Investigación e Innovación Agroalimentaria (CIAGRO-UMH), Miguel Hernández University of Elche, Elche, Spain **3** Department of Didactics on Experimental Sciences, Faculty of Education, University of Murcia, Murcia, Spain **4** University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic **5** MARE – Centro de Ciências do Mar e do Ambiente, Departamento de Paisagem, Ambiente e Ordenamento, Escola de Ciências e Tecnologia, Universidade de Évora, Évora, Portugal **6** Instituto de Biodiversidad y Medioambiente (BIOMA), Universidad de Navarra, Pamplona, Spain **7** Departamento de Biología Vegetal y Ecología, Facultad de Farmacia, Universidad de Sevilla, Sevilla, Spain **8** Departamento de Zooloxía, Xenética e Antropoloxía Física, Facultade de Bioloxía, Universidade de Santiago de Compostela, A Coruña, Spain **9** MARE – Centro de Ciências do Mar e do Ambiente, Faculdade de Ciências

da Universidade de Lisboa, Lisboa, Portugal **10** Instituto Pirenaico de Ecología (IPE), CSIC, Zaragoza, Spain **11** GRECO, Institute of Aquatic Ecology, University of Girona, Girona, Spain **12** Sistemática de Plantas Vasculares, Real Jardín Botánico (RJB-CSIC), Madrid, Spain **13** Departamento de Biodiversidad, Ecología y Evolución, Universidad Complutense de Madrid, Madrid, Spain **14** Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal **15** Department of Basic Medical Sciences, School of Medicine, Universidad San Pablo-CEU, CEU Universities, Madrid, Spain **16** Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, Oviedo, Asturias, Spain **17** Asociación Herpetológica Española (A.H.E.), Museo Nacional de Ciencias Naturales (MNCN-CSIC), Madrid, Spain **18** Department of Plant Biology and Ecology, University of the Basque Country UPV/EHU, Bilbao, Spain **19** Department of Biogeographical Ecology and Evolution, Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Faro, Portugal **20** Tragsatec, TSUP Planificación y Gestión Hídrica, Grupo Tragsa – SEPI, Madrid, Spain **21** GEIB - Grupo Especialista en Invasiones Biológicas, León, Spain **22** Centre of Geographical Studies, Institute of Geography and Spatial Planning, University of Lisbon, 1600-276 Lisboa, Portugal **23** Associated Laboratory Terra, Tapada da Ajuda, 1349-017 Lisboa, Portugal **24** Coordinador de Lista Vermelha da Flora de Portugal Continental, Sociedade Portuguesa de Botânica, Coimbra, Portugal **25** Departament de Ciència Animal, Universitat de Lleida, Lleida, Spain **26** Centre Tecnològic Forestal de Catalunya (CTFC), Solsona, Lleida, Spain **27** Ecología, conservación de macrófitos acuáticos y cambio global, Real Jardín Botánico (RJB-CSIC), Madrid, Spain **28** Departamento de Biología de la Conservación, Estación Biológica de Doñana (EBD-CSIC), Sevilla, Spain **29** Departamento de Ecología y Gestión Costera, Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC), Cádiz, Spain **30** Técnico Superior en Medio Ambiente, VAERSA-GVA, Generalitat Valenciana, Valencia, Spain **31** CCMAR - Centro de Ciências do Mar, Universidade do Algarve, Algarve, Portugal **32** Departamento de Zoología, Universidad de Córdoba, Córdoba, Spain **33** AZTI, Investigación Marina, Gestión Ambiental de Mares y Costas, Pasaia, Guipúzkoa, Spain **34** Departamento de Ecología e Hidrología, Facultad de Biología, Universidad de Murcia, Murcia, Spain **35** Associació Catalana de Malacologia-Museu Blau., Barcelona, Spain **36** Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales (MNCN-CSIC), Madrid, Spain **37** Departamento de Biología (Botánica), Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain **38** Sorelló - Estudis al Medi Aquàtic, Girona, Spain **39** Departamento de Zoología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain **40** Consejería de Sostenibilidad, Medio Ambiente y Economía Azul, Junta de Andalucía, Huelva, Spain **41** Departamento de Ecología de Humedales, Estación Biológica de Doñana (EBD-CSIC), Sevilla, Spain **42** SIBIC, Departament de Ciència Animal, Universitat de Lleida, Lleida, Spain

Corresponding authors: Jose M. Zamora-Marín (josemanuel.zamora@um.es); Francisco J. Oliva-Paterna (fjoliva@um.es)

Abstract

Aquatic ecosystems are particularly vulnerable to the introduction of non-indigenous species (NIS), leading to multi-faceted ecological, economic and health impacts worldwide. The Iberian Peninsula comprises an exceptionally biodiverse Mediterranean region with a high number of threatened and endemic aquatic species, most of them strongly impacted by biological invasions. Following a structured approach that combines a systematic review of available information and expert opinion, we provide a comprehensive and updated multi-taxa inventory of aquatic NIS (fungi, macroalgae, vascular plants, invertebrates and vertebrates) in Iberian inland waters. Moreover, we assess overall patterns in the establishment status, introduction pathways, native range and temporal introduction trends of listed NIS. In addition, we discuss the legal coverage provided by both national (Spanish and Portuguese) and European NIS regulations. We inventoried 326 aquatic NIS in Iberian inland waters, including 215 established, 96 with uncertain establishment status and 15 cryptogenic taxa. Invertebrates (54.6%) and vertebrates (24.5%) were the groups with the highest number of NIS, with Arthropoda, Mollusca, and Chordata being the most represented phyla. Recorded NIS originated from diverse geographic regions, with North and South America being the most frequent. Vertebrates and vascular plants were mostly introduced through intentional pathways (i.e. release and escape), whereas invertebrates and macroalgae arrived mostly through unintentional ways (i.e. contaminant or stowaway). Most of the recorded NIS were introduced in Iberian inland waters over the second half of the 20th century, with a high number of NIS introductions being reported in the 2000s. While only 8% of the recorded NIS appear in the European Union list of Invasive Alien Species of Union concern, around 25% are listed in the Spanish and Portuguese NIS regulations. This study provides the most updated checklist of Iberian aquatic NIS, meeting the requirements set by the EU regulation and providing a baseline for the evaluation of its application. We point out the need for coordinated transnational strategies to properly tackle aquatic invasions across borders of the EU members.

Keywords

Alien species, checklist, environmental management, estuaries, inland waters, Portugal, regulation, Spain, Western Mediterranean

Introduction

Compared to terrestrial and marine ecosystems, freshwater and transitional waters (hereafter collectively referred to as inland waters) are especially vulnerable to biological invasions due to their intrinsic environmental features (Moorhouse and Macdonald 2015; McFadden et al. 2023), and the high introduction pressure promoted by the wide range of human activities developed in these aquatic habitats (Reid et al. 2019; Cabral et al. 2020). For instance, inland waters support commercial fisheries, aquaculture, shipping, and diverse recreational activities (e.g. sport fishing or navigation). In addition, inland waters are subject to different human infrastructures such as dams, ditches or water transfer systems (Ojaveer et al. 2018; Anastácio et al. 2019; Bailey et al. 2020). Such human activities are well-known drivers of the introduction and spread of non-indigenous species (hereafter, NIS) (Nunes et al. 2015). As a consequence, there is a growing evidence of major impacts caused by NIS at multiple ecological levels in inland waters, with well-demonstrated detrimental effects on native aquatic biota, ecosystem functions and services (Vilà et al. 2011; Gallardo et al. 2016; Guareschi

et al. 2021). Under this scenario, management actions are urgently required to slow down the introduction rate of NIS in inland waters, to control populations of already established NIS and to prevent secondary spread towards still non-invaded aquatic ecosystems (Britton et al. 2023). In this context, updated inventories of NIS and comprehensive assessments on introduction pathways, native regions and temporal trends of introductions are fundamental for elucidating the causes and consequences of the invasion process (Seebens et al. 2017; Fuentes et al. 2020). Moreover, NIS management policies should be based on a transnational approach involving coordinated surveillance efforts (Bailey et al. 2020; Capinha et al. 2023). This is especially relevant when neighbouring countries share river basins and, consequently, aquatic ecosystems are largely interconnected. However, legislation and direct management are nowadays mostly implemented at national scale (Anastácio et al. 2019).

The Mediterranean basin is one of the major global hotspots of biodiversity, with the Iberian Peninsula comprising a particularly species-rich area and harbouring high numbers of endemic species (Araújo et al. 2007; Buira et al. 2017; Rosso et al. 2018). This is even more evident in the case of the Iberian aquatic biodiversity, which shows outstanding ratios of species singularity and endemicity (Doadrio et al. 2011; Hermoso et al. 2016). For instance, about 80% of freshwater fish, 40% of amphibian and 25% of water beetle species occurring in the Iberian Peninsula are endemic (Doadrio et al. 2011; Hermoso et al. 2016). However, most of these species are highly threatened and particularly vulnerable to NIS introductions (e.g. Cruz et al. 2008; Ruiz-Navarro et al. 2013; Romero 2015). In fact, the introduction of both animal and plant species in Iberian inland waters is a long-lasting and ongoing process, whose adverse effects have been largely documented from an ecological (Aguiar and Ferreira 2013; Anastácio et al. 2019), socioeconomic (Durán et al. 2012; Angulo et al. 2021) and public health (Collantes et al. 2015; Sánchez et al. 2021) perspective. In recent years, a few studies have provided reference NIS inventories and first assessments targeting different inland ecosystems (freshwater or transitional environments) and biotic groups, at Iberian, national (Spanish or Portuguese) and regional scales. For instance, García-Berthou et al. (2007) provided the first checklist of animal species naturalised in Iberian inland waters, and Cobo et al. (2010) provided a similar inventory for Galicia (Spain), adding plant species and comparing with other areas from the Iberian Peninsula. Aguiar and Ferreira (2013) conducted an overview of the available knowledge on invasive plants in Iberian rivers, whereas Rodríguez-Merino et al. (2017) focused on the potential distribution of non-native aquatic macrophytes in Iberian inland waters. Later, Anastácio et al. (2019) compiled records of animal NIS introduced across Portuguese freshwater ecosystems and provided a thorough assessment of the temporal introduction rate, native regions, reported impacts and legal coverage from both Portuguese and European lists of NIS. Muñoz-Mas and García-Berthou (2020) conducted a comprehensive review of aquatic non-indigenous fauna introduced in Iberian inland waters and compared temporal introduction rates between the Iberian Peninsula and two Iberian subregions (Portugal and Galicia). More recently, through a horizon scan exercise, Oficialdegui et al. (2023) have identified the most relevant NIS recorded or potential introductions to Iberian inland waters, but a relevant number of NIS that did not score high enough are missing from this list.

Most of the above-mentioned studies retrieved NIS records exclusively from published scientific literature, thus overlooking grey literature and unpublished but validated NIS records from private inventories, institutional repositories and official databases. Because of time lags between detecting a NIS in the field and its corresponding publication (Zenetos et al. 2017), official online databases are currently essential resources for regularly updating NIS checklists and informing management policies more rapidly. Overall, NIS records concerning Iberian inland waters are scattered across several publications and data sources, thus posing a serious constraint for an integrated NIS management at different spatial scales. Moreover, they are often biased towards animal taxa and purely freshwater ecosystems, so studies concerning aquatic plants (e.g. Aguiar and Ferreira 2013) and transitional waters (Zorita et al. 2013; Cabral et al. 2020; Zamora-Marín et al. 2023) are scarce and conducted at local or regional scales. Hence, multi-taxa inventories of NIS introduced in Iberian inland waters (both fresh and transitional waters) are needed, since they comprise key tools in decision-making with potential implications on NIS regulation policies at national (Spanish and Portuguese) and European levels (e.g. the European Union list of Invasive Alien Species of Union concern, hereafter "the Union list"). In fact, the EU regulation No 1143/2014 on the prevention and management of the introduction and spread of invasive alien species (IAS) recommends the EU Member States to provide updated checklists on NIS introduced in their territory and conduct a comprehensive analysis of the unintentional introduction and spread pathways of the IAS of Union concern (Piria et al. 2018).

This study updates the information on aquatic NIS occurring in Iberian inland waters by combining knowledge from a diverse panel of experts with an extensive screening of published literature (both international and grey literature), online databases (e.g. GBIF, EASIN and CABI), and technical reports or off-line databases provided by environmental agencies. Through a broad multi-taxa approach including fungi, flora and fauna, this study aims (1) to provide an extensive and updated inventory of NIS introduced in Iberian inland waters, (2) to assess overall patterns in introduction pathways, native regions, and temporal introduction rates, and (3) to discuss the legal coverage of national (Spanish and Portuguese) and European IAS regulation. To make this study as robust as possible, this comprehensive assessment was conducted by an expert-consensus-based approach, which ensured a reliable checklist validation from a taxonomic and state-of-the-art viewpoint, since misidentification or distributional errors are common when no group-specific experts are involved in NIS multi-taxa studies (Zenetos et al. 2017).

Methods

Study area and target habitats

The Iberian Peninsula is mostly comprised of the mainland territory of Spain and Portugal. This area is characterised by a wide climatic gradient which extends from the northwestern (temperate oceanic conditions expressed as high rainfall and humidity values, and low continentality) to the southeastern edge (Mediterranean semiarid

conditions), including also large parts of the territory exposed to Mediterranean climate with higher continentality. The coastline of the Iberian Peninsula extends over 3,904 km across the Mediterranean Sea (1,670 km), the Atlantic Ocean (1,367 km) and the Cantabrian Sea (867 km). Most of the Iberian territory is framed within major river basins, some of them shared between Spain and Portugal (e.g. Guadiana, Tagus and Douro catchments). Following the European Water Framework Directive (hereafter, WFD) (EC 2000), we considered inland waters as those standing or flowing surface aquatic ecosystems (both fresh and transitional waters) placed across land boundaries. Hence, this term included typically lotic (i.e. rivers and streams) and lentic freshwater ecosystems (i.e. lakes, wetlands and reservoirs), small water bodies (i.e. ponds and pools) and transitional or estuarine aquatic systems influenced by freshwater inputs (i.e. marshlands, brackish waters, estuaries and coastal lagoons). Here, all these aquatic ecosystems were collectively considered and referred to as inland waters. Inland waters from the Balearic and Macaronesia (i.e. Canary Islands, Madeira and the Azores archipelagos) islands were excluded.

Compiling records and attributes of NIS

An integrative and structured approach based on multiple data sources was applied to generate a comprehensive up-to-date inventory of all aquatic NIS occurring in Iberian inland waters. Firstly, we compiled all available literature on NIS occurrence in Iberian inland waters, including articles published in indexed international journals, grey literature (e.g. articles in regional journals or bulletins), online databases and technical reports. For peer-reviewed literature, we made a query in the Web of Science to retrieve all potential publications focused on NIS in Iberian inland waters. Boolean search terms included all words related to NIS or potential synonyms (i.e. alien, allochthonous, exotic, introduced, invasive, non-native and non-indigenous), target environments (i.e. freshwater, transitional, reservoir/s, lake/s, pool/s, pond/s, river/s, stream/s, estuary/ies and coastal lagoon/s) and the study area (i.e. Iberia, Iberian Peninsula, Spain and Portugal). Resulting publications were screened to generate a list of NIS introduced in Iberian inland waters. This preliminary list of NIS was further complemented with records from grey literature, national technical reports and regional checklists, as well as from the following databases: the European Alien Species Information Network (EASIN; http://easin.jrc.ec.europa.eu), CABI´s Invasive Species Compendium (CABI-ISC; http://www.cabi.org/isc/), the Global Invasive Species Database (GISD; www.iucngisd.org), the EXOCAT database (http://exocatdb.creaf.cat/base_dades/#), the AquaNIS database (http://www.corpi.ku.lt/databases/aquanis/) and the Global Biodiversity Information Facility database (GBIF; http://www.gbif.org/). We recorded all aquatic NIS introduced in Iberian inland waters up to August 2022.

We considered target taxa to be all those NIS able to live in freshwater and/or transitional waters at least during part of their life cycle. Aquatic taxa native from a given Iberian river basin but introduced in other Iberian catchments (i.e. translocated species) were excluded from our inventory. This preliminary list was agreed and validated

by a panel of 65 experts in conservation biology and invasion science from Spain and Portugal, covering both types of target aquatic ecosystems (freshwater and transitional environments) and all biotic groups potentially containing aquatic NIS.

Following previous studies (see Muñoz-Mas and García-Berthou 2020), taxa clearly introduced into Iberian inland waters with self-sustaining populations were classified as "established" (most commonly referred as "naturalized" in plants), whereas those non-indigenous taxa reported to occur in the study area but without known self-sustaining populations were classified as "uncertain" (most commonly referred as "casual" in plants). Taxa with unclear biogeographic history in the Iberian Peninsula (i.e. native/introduced status) were considered as "cryptogenic". The recorded aquatic NIS were classified into five major biotic groups: vertebrates, invertebrates (both free-living and symbionts), vascular plants, macroalgae and fungi. From the screened data sources, we searched and retrieved the following four relevant species-specific attributes for all recorded NIS: native regions, introduction pathways, year of introduction and functional group. Native regions for the recorded NIS were divided into nine geographic regions: Africa, Antarctica, temperate Asia, tropical Asia, Australasia, Europe, Pacific Ocean, North America, and South America. According to the Convention of Biological Diversity (CBD 2014) and as stated by the EU regulation (European Commission 2017), which complemented the classification of introduction pathways previously proposed by Hulme et al. (2008), we used the following seven major categories to characterise the introduction pathways: Release, Escape, Contaminant, Stowaway, Corridor, Unaided and Unknown. Whenever possible, the most probable introduction pathways were based on published literature for the Iberian Peninsula.

For each recorded NIS, the year of introduction (i.e. first detection in the wild) in Europe and both Iberian countries was obtained. This date at European scale was mostly retrieved from EASIN, whereas at the national scale (for Spain and Portugal) was mainly retrieved from scientific literature providing first records for the Iberian Peninsula. When unreported in the literature, we applied a conservative approach and considered the year of the corresponding publication as the year of introduction, following Cobo et al. (2010) and Muñoz-Mas and García-Berthou (2020). In the case of host-specific alien invertebrate symbionts (e.g. *Onchocleidus dispar*), we considered the year of introduction to be that of the host. By contrast, in the case of generalist non-indigenous parasites (e.g. *Lernaea cyprinacea*), which can be introduced with many host species (native or non-indigenous species), we considered the first detection year of the parasite. Additionally, we retrieved from EASIN the name of the country/ies where a given NIS was detected for the first time within Europe. Recorded NIS were also classified into the following nine functional groups: primary producers, herbivores, predators or parasites, detritivores, filter feeders, omnivores, xylophages, pollinators and polyphagous. Lastly, we screened the current regulation to assess the legal coverage of the recorded NIS. In particular, we checked the inclusion of NIS in the Union list, the Spanish IAS Catalogue (Royal Decree 630/2013, latest update 1 December 2020), the Spanish Allochthonous List (Royal Decree 570/2020), and the Portuguese List of IAS (Decree-Law 92/2019).

Data analyses

We analysed which native regions, introduction pathways and functional groups were most prevalent for the recorded NIS. To avoid overrepresentation of those NIS associated with two or more categories, data on these attributes were down-weighted in frequency-related analyses following the strategy of Muñoz-Mas and García-Berthou (2020). We used data on the first year of introduction of the recorded NIS in Europe, Spain and Portugal to compare temporal trends. We also applied linear models to assess pairwise differences in introduction dates among both Iberian countries and Europe (e.g. Spain vs Europe) and to determine potential NIS introduction delays. Lastly, legal coverage of the listed NIS in the official European, Spanish and Portuguese regulation lists of NIS was visually assessed through Venn diagrams, obtained with the package VennDiagram (Chen 2022), implemented in the free software R v.4.0.3 (R Core Team 2022).

Results

Taxonomic approach

We recorded 326 non-indigenous taxa in Iberian inland waters, which included 215 clearly established, 96 uncertain and 15 cryptogenic taxa (Suppl. material 1). The listed aquatic NIS represented five major biotic groups, with invertebrates (54.6% of total taxa) being the dominant one, followed by vertebrates (24.5%), vascular plants (12.6%), macroalgae (7.4%) and fungi (0.9%). These aquatic NIS covered virtually all phyla (15) inhabiting Iberian inland waters, and belonged to 36 classes. The most represented phyla (or division in the case of plants) were Chordata (27.0%), Arthropoda (20.6%) and Mollusca (16.0%), whereas Magnoliophyta (10.7%), Rhodophyta (6.1%), Platyhelminthes (5.5%) and Annelida (5.2%) gathered a lower number of NIS, and the remaining phyla showed marginal values (≤ 2%) (Fig. 1). Overall, the ratio established/total NIS was congruent across all biotic groups, with the species-richest phyla having a greatest number of established NIS (range 47–94% of established taxa from the total NIS richness). At lower taxonomic resolution, Actinopterygii (14.1%) was the class with most species among all the taxa recorded, followed by Magnoliopsida (10.7%), Malacostraca (9.2%), Gastropoda (8.3%), Bivalvia (7.4%) and Florideophyceae (6.1%) (Suppl. material 2: fig. S1).

Most of the aquatic non-indigenous vertebrates (57.5%) were fish (Class Actinopterygii) and they mainly corresponded to NIS well established in Iberian inland waters (34 established; 12 uncertain taxa), with Cyprinidae being the dominant among the 16 recorded families (16 cyprinids out of 46 listed non-indigenous fish species). Reptiles were the second species-richest class among vertebrates (13 NIS, 16.2% of vertebrates) and they were exclusively represented by freshwater turtle species, with only one taxon being clearly established (*Trachemys scripta*). A similar pattern was found in birds and amphibians, with eight listed NIS for both classes but only two species of birds (*Alopochen aegyptiaca* and *Cairina moschata*) and three species of amphibians
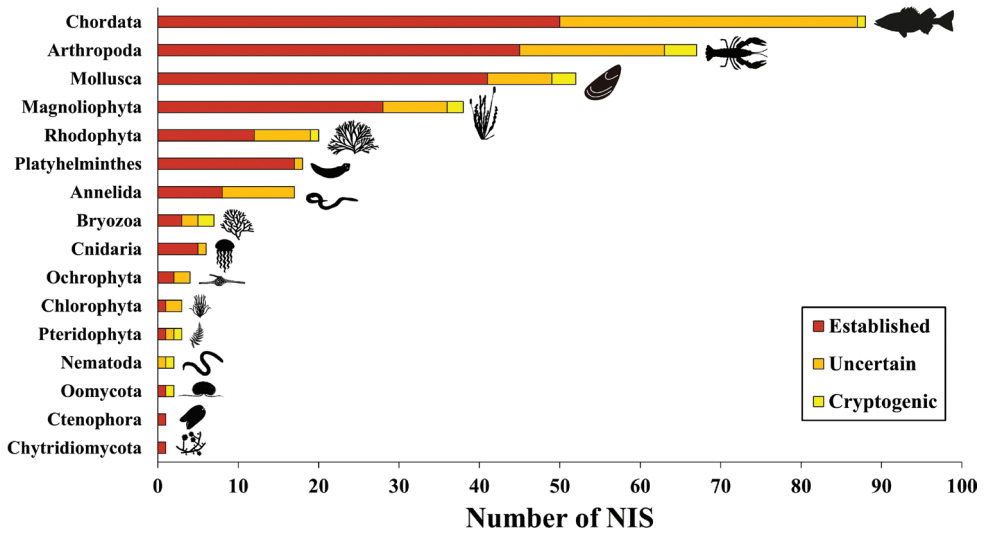
Figure 1. Cross-group richness of aquatic non-indigenous species (NIS) recorded in inland waters (including freshwater and transitional waters) from the Iberian Peninsula. Groups correspond to phyla (animals) or divisions (plants). Colours refer to the proportion of NIS belonging to each establishment stage (established, uncertain or cryptogenic). From top to bottom, groups are ranked from the species-richest to the species-poorest.

(*Discoglossus pictus*, *Pelophylax* kl. *grafi* and *Xenopus laevis*), respectively, were considered as established. On the other hand, the recorded non-indigenous invertebrates were represented by a widely diversified set of species that corresponded to 24 classes including 62 orders. Podocopida (19 NIS) and Decapoda (16 NIS) were the invertebrate orders with most species. Regarding vascular plants, our inventory included submerged, floating and emergent aquatic plants occurring in Iberian inland waters, which generally corresponded to hydrophytes and helophytes. Magnoliopsida (35 NIS) was the dominant group of vascular plants, 12 of these species belonging to the order Alismatales, whereas the class Polypodiopsida hosted three non-indigenous pteridophytes. Among macroalgae, Rhodophyta was the dominant group (20 NIS), whereas Ochrophyta (4 NIS) was much less represented. Lastly, non-indigenous fungi species (3 NIS) were exclusively represented by pathogens belonging to the genera *Batrachochytrium* and *Aphanomyces*, which mostly affect amphibians and crayfish, respectively.

Native regions

Native regions of the recorded NIS corresponded to all geographic areas, with the exception of Antarctica (Fig. 2). North America (26.8%) and temperate Asia (18.8%) were the most common native regions of Iberian aquatic NIS. We found 197 NIS (60.8%) that were native to a single geographic region (Suppl. material 2: fig. S2a). Overall, NIS belonging to all biotic groups were native to a wide variety of geographic regions (Fig. 2). Particularly, vertebrates were mostly native to North America, Europe and temperate Asia, whereas invertebrates were native to all geographic regions and they comprised
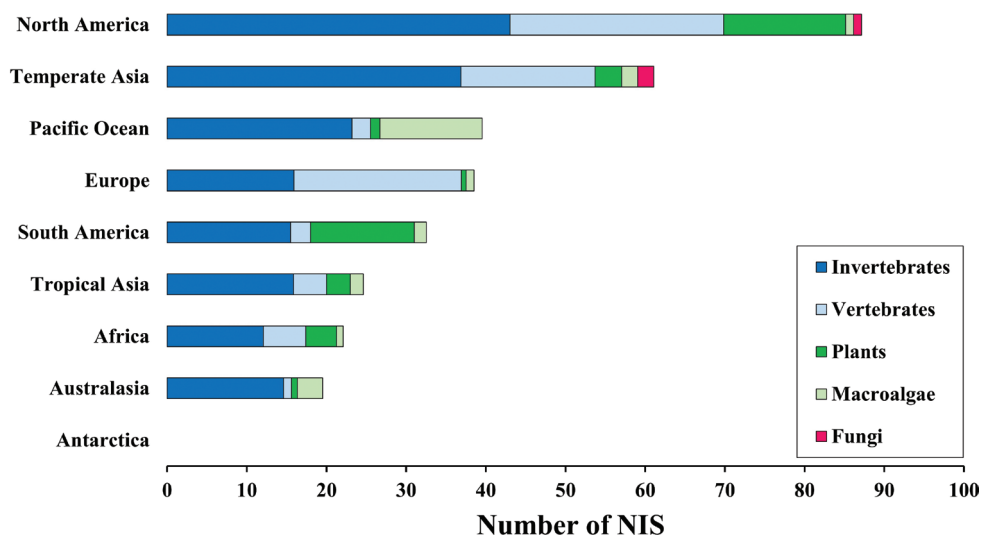
Figure 2. Native regions for the aquatic non-indigenous species (NIS) recorded in inland waters (both freshwater and transitional waters) from the Iberian Peninsula. Results are displayed according to the five main biotic groups considered. As several NIS presented two or more native regions, data were down-weighted to avoid overrepresentation.

about half of the NIS considered as native to each region. Vascular plants were mainly native to North and South America, whereas most non-indigenous macroalgae were native to the Pacific Ocean. Fungi were native from temperate Asia and North America.

Pathways of introduction

We identified four major pathways as responsible of NIS introductions in Iberian inland waters, which totalled about 90% of the recorded taxa: Stowaway (26.1%), Contaminant (25.6%), Escape (21.2%) and Release (17.1%) (Suppl. material 2: fig. S3). The vast majority of recorded NIS were introduced through a single pathway (170 NIS, 53.8%) or two pathways (127 NIS, 40.2%) (Suppl. material 2: fig. S2b). This pattern in the number of introduction pathways was homogenous across biotic groups. Taxonomic-related patterns of NIS arrival were observed across major biotic groups (Fig. 3). For instance, non-indigenous invertebrates and macroalgae arrived mostly through stowaway and contamination (i.e. unintentional pathways), whereas vertebrates and vascular plants were mainly intentionally introduced through escape and release.

Timeline of NIS introduction

Year of introduction was available for most of the recorded NIS (283/326 for Europe, 280/305 for Spain, and 151/178 for Portugal), thus ensuring representative data on NIS introduction to ascertain temporal arrival rates. From the 1860s to 1960s, the
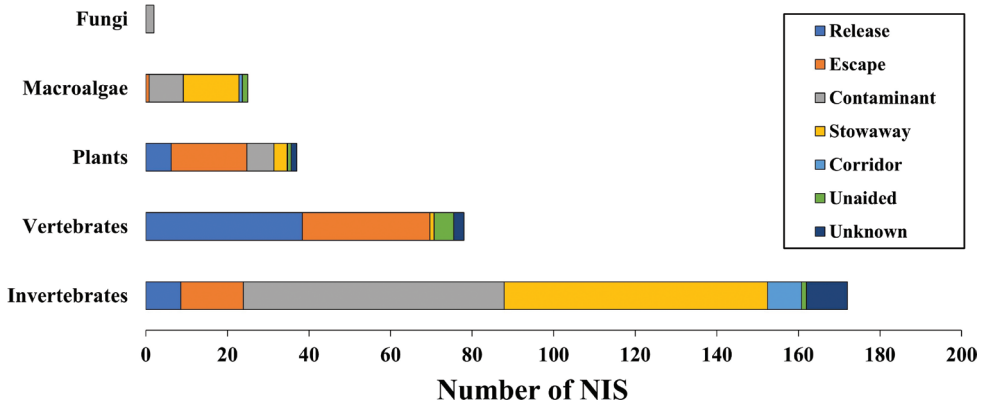
Figure 3. Contribution of the categories of introduction pathways to the arrival of aquatic non-indigenous species (NIS) to inland waters (including freshwater and transitional waters) from the Iberian Peninsula. NIS are grouped into the five major biotic groups considered. As several NIS were introduced through two or more pathways, data were down-weighted to avoid overrepresentation of these categories.
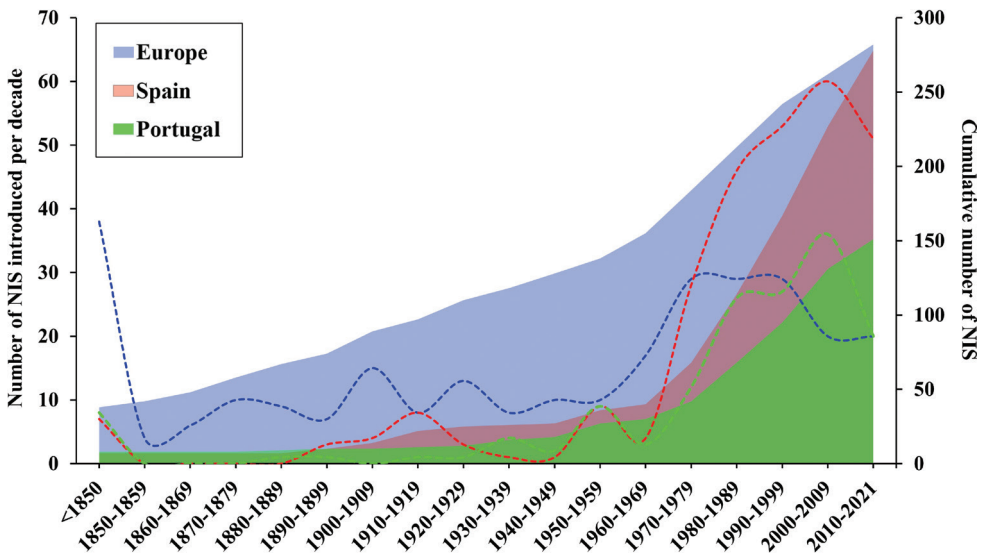

Figure 4. Temporal introduction rates of aquatic non-indigenous species (NIS) recorded in inland waters (including freshwater and transitional waters) from the Iberian Peninsula. Filled areas represent the cumulative number of introduced NIS in European, Spanish and Portuguese inland waters, whereas lines represent the decadal pace of NIS introduction. Note that the last decade includes two additional years (2020–2021) to allow for reliable data representation.

recorded NIS were introduced in European inland waters at a pace of 5–15 species per decade, reaching introduction rates of 30 species per decade over the end of the 20th century (1970s–2000s), though this pace has slightly decreased in the past two decades (Fig. 4). In both Spain and Portugal, some widespread NIS were introduced before the

1850s, such as the common carp (*Cyprinus carpio*), the goldfish (*Carassius auratus*) and the tadpole snail (*Physella acuta*). On the other hand, a clear temporal variation was observed in the contribution of each introduction pathway to the arrival of the recorded NIS to Iberian inland waters (Suppl. material 2: fig. S4). For instance, intentional pathways (i.e. Release and Escape) made a higher contribution to the arrival of aquatic NIS to Iberian inland waters before 1950, whereas unintentional pathways (i.e. Contaminant and Stowaway) gained relevance during the second half of the 20th century.

The delay in aquatic NIS introductions among the three regions (Europe, Spain and Portugal) was only evident for pairwise comparisons between national and continental scales (Fig. 5), with no significant differences being observed between both Iberian countries (Fig. 5c). Results from linear models conducted separately across taxonomic groups supported the similar pace of NIS introduction among both countries, particularly in the case of invertebrates ($R^2 = 0.5168$) and vertebrates ($R^2 = 0.8497$). When compared to the year of introduction in Europe (Fig. 5a, b), both Spain and Portugal showed a

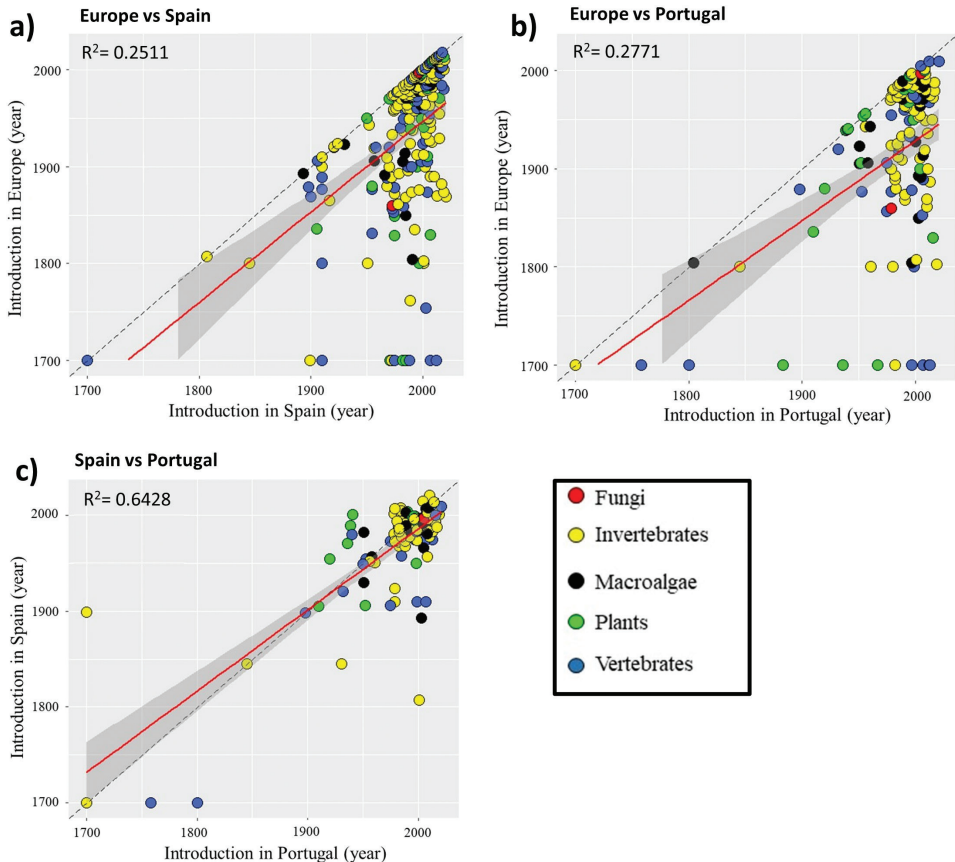**Figure 5.** Scatterplots and linear regression functions (red line) of the year of introduction of aquatic non-indigenous species (NIS) in three regions: Europe vs Spain (a), Europe vs Portugal (b) and Spain vs Portugal (c). Each dot represents a given NIS, with colour indicating the five considered biotic groups. Dashed lines represent the equality line and grey shadow correspond to confidence intervals.

similar pattern in the delay of NIS introductions. This situation was particularly evident after the 1900s, when both countries received new NIS with an average delay of about 50 years after their introduction in European inland waters. Interestingly, both Spain and Portugal comprised major countries of first detection in Europe (i.e. European countries acting as gateways for aquatic NIS introduction at continental scale) to a large number of the recorded NIS. For instance, at a continental scale, 51 NIS were firstly detected in Spain and 22 in Portugal, whereas UK (38 NIS), France (35) and Italy (31) were also relevant countries of first introduction in Europe (Suppl. material 2: fig. S5).

Functional groups

The recorded NIS spanned a wide variety of functional groups (Suppl. material 2: fig. S6), but they were mostly represented by predators (26.4%), filter-feeders (24.7%), primary producers (20.1%) and omnivores (18.5%). No alien pollinator species were detected, whereas the cryptogenic ship worm (*Teredo navalis*) was reported as the single xylophagous (i.e. wood-eating) species inhabiting Iberian inland waters.

Legal coverage of NIS regulation

Only 26 (8.0%) out of the 326 recorded NIS are included in the Union List of the EU Regulation (Fig. 6). Both national lists of IAS provided a higher legal coverage, since 86 (26.4%) and 80 (24.5%) out of the total recorded NIS were included in the Portuguese and Spanish lists of IAS, respectively. Although both national lists shared two thirds of the listed NIS, we found a clear regulation mismatch between them. For instance, the Portuguese list of IAS did not include 15 Spanish-listed taxa despite being
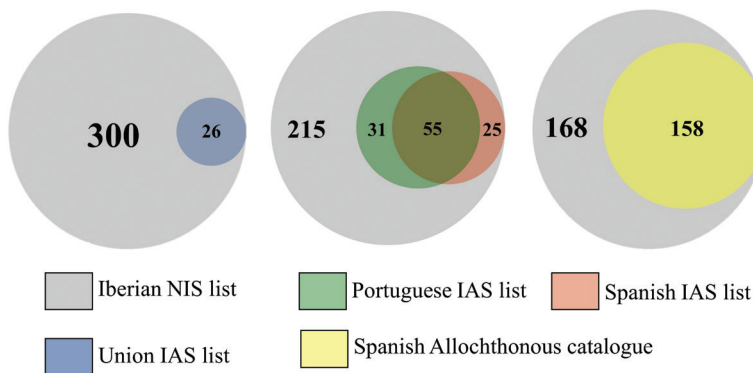
Figure 6. Venn diagrams representing the legal coverage of the official regulation lists for aquatic non-indigenous species (NIS) in Europe, Spain and Portugal. Large circles represent the pool of 326 NIS introduced in Iberian inland waters (Iberian NIS list), whereas smaller circles represent the number of aquatic Iberian NIS which are listed in the Union IAS list, in the Portuguese IAS list, in the Spanish Catalogue of Invasive Alien Species (Spanish IAS list) and in the list of allochthonous species able to impact on Spanish native biodiversity (Spanish Allochthonous catalogue). The number of NIS exclusive to and shared by each list is indicated within circles.

also introduced in Portugal, and 29 out of the 31 Portuguese-listed were not considered in the Spanish regulation. The highest legal coverage was provided by the Spanish allochthonous catalogue, since it included 158 out of the 326 recorded NIS (48.5%). Some taxonomic groups were clearly underrepresented in the European and national regulation lists (Suppl. material 2: fig. S7). For instance, none of the 52 recorded non-indigenous mollusc species were included in the Union list, and no alien platyhelminthes were included in any of the official lists of IAS (EU, Spanish and Portuguese). Chordata and Magnoliophyta were always the best represented ones in NIS regulations, whereas Arthropoda and Mollusca were comparatively the least considered.

Discussion

Checklist of NIS in Iberian inland waters

Our multi-taxa assessment provides the most updated and comprehensive inventory of NIS occurring in freshwater and transitional waters from the Iberian Peninsula (mainland Spain and Portugal). By gathering expert knowledge, published literature and other available data sources, we recorded 326 taxa of fungi, macroalgae, vascular plants, invertebrates and vertebrates already introduced and detected in Iberian inland waters, including established, uncertain and cryptogenic taxa. As compared to other reference checklists (Table 1), our multi-taxa inventory supports the occurrence of 258 aquatic animals and 41 plants introduced in Iberian inland waters, which is twice the number of NIS provided by previous reference studies (Rodríguez-Merino et al. 2017; Muñoz-Mas and García-Berthou 2020). These differences in NIS richness are likely due to the fact that former assessments were exclusively based on partial accounts of the available evidence (i.e. only NIS records published in international literature). Here, by combining information from multiple data sources (i.e. published international and grey literature, official online databases and technical reports) and looking for consensus among a widely diversified panel of Iberian experts, we achieved the most reliable and updated NIS checklist. In this context, official data repositories have emerged in recent years as essential tools to periodically update NIS checklists and assist management actions (Katsanevakis et al. 2014). Hence, online open-access databases provide a source of NIS records complementary to published literature, which suffers from long lag times that occur from field NIS detection to publication (Zenetos et al. 2017). Our integrative approach ensures a comprehensive assessment that will optimally support prioritising actions on NIS management (Katsanevakis et al. 2014).

In addition, most of the reference checklists (Table 1) focused only on non-indigenous fauna and/or freshwater environments as target systems, with NIS inventories on aquatic flora or transitional waters being much more limited. As stated here, information on NIS occurrence in Iberian inland waters is notably scattered across different data sources, which may place constraints on prospective data analysis and

implementation of management actions by national or regional governments and river basin authorities. Moreover, the lack of integrated studies at national or regional scales precludes the assessment of global patterns and correlates in NIS introduction (Lonsdale 1999; Vilà et al. 2001). Therefore, our multi-taxa assessment is particularly valuable from a management viewpoint, because it unifies scattered NIS records and provides an updated inventory of aquatic NIS established (or potentially established) in Iberian inland waters, as well as includes a freely available database containing relevant species-specific information (Suppl. material 1). However, this inventory is likely to be subject to potential taxonomic biases derived from knowledge gaps of some poorly-known taxa, because of various biotic groups that are especially diverse and able to thrive in inland waters (e.g. annelids, nematodes, flatworms or chlorophytes) were underrepresented in our checklist. To date, research efforts focused on such biotic groups in Iberian inland waters have been sparse and scattered, which could have limited the number of recorded NIS. Through an expert-consensus-based approach, our study likely reduces the risk of taxonomic uncertainties typically occurring during the process of listing invasive species (McGeoch et al. 2012), thus providing a reliable and valuable list for environmental agencies, policy-makers and conservationists.

Table 1. Number of non-indigenous species (NIS) reported by the main previous studies providing reference checklists in the study area or related geographical regions. NA means no data available.

Reference checklist	Target environments	Target taxa	Study area	N established	N uncertain	N cryptogenic	N total
This study	Freshwater and transitional	Fungi, macroalga, plants, and animals	Iberian Peninsula	215	96	15	326
Zamora-Marín et al. 2023	Transitional	Animals	Spanish Mediterranean coast	93	30	6	129
Oficialdegui et al. 2023	Freshwater and transitional	Plants and animals	Iberian Peninsula	103	21	2	126
Muñoz-Mas and García-Berthou 2020	Freshwater and transitional	Animals	Iberian Peninsula	125	18	6	149
Anastácio et al. 2019	Freshwater	Animals	Mainland and insular Portugal	67	NA	NA	67
Rodríguez-Merino et al. 2018	Freshwater	Vascular plants	Europe	NA	NA	NA	60
Gofas et al. 2017	Marine	Molluscs	Mainland and insular Spain	36	NA	2	38
Rodríguez-Merino et al. 2017	Freshwater	Macrophytes	Iberian Peninsula	20	NA	NA	20
Chainho et al. 2015	Marine and transitional	Algae, plants and animal	Mainland and insular Portugal	78	46	NA	133
Aguiar and Ferreira 2013	Freshwater (rivers) and riparian	Plants	Iberian Peninsula	NA	NA	NA	NA
Cobo et al. 2010	Freshwater	Animals	Iberian Peninsula	NA	NA	NA	78
Sanz-Elorza et al. 2001	Terrestrial and freshwater	Plants	Spain	NA	NA	NA	176

Patterns in aquatic NIS introduction

Aquatic non-indigenous vertebrates and invertebrates were generally native to North America and temperate Asia, though a relevant proportion of taxa were also originated from Europe, being all these patterns congruent with previous studies (Anastácio et al. 2019; Muñoz-Mas and García-Berthou 2020). For vertebrates, this pattern was mainly due to the high number of non-indigenous fish species native to North America, Asia and Europe, which have been intentionally introduced (i.e. released) in Iberian inland waters to promote recreational fishing (García-Berthou et al. 2007). Most of these introduced fish corresponded to large piscivorous species (e.g. *Micropterus salmoides* and *Esox lucius*) and small-sized fish (e.g. *Alburnus alburnus* and *Abramis bjoerkna*) used as forage species for non-indigenous piscivores (Elvira and Almodóvar 2001). A non-negligible number of non-indigenous fish have also been intentionally released for ornamental purposes (e.g. *Carassius auratus*) or as a consequence of the aquarium trade (e.g. *Aphanius fasciatus*, *Poecilia reticulata* and *Misgurnus bipartitus*) (Maceda-Veiga et al. 2013; Clavero et al. 2023), whereas few of them became naturalised through escapes from fish farm facilities (e.g. *Ictalurus punctatus*) (Elvira and Almodóvar 2001; Maceda-Veiga et al. 2013). Non-indigenous fish introduced in Iberian inland waters but native to Europe are of particular interest because they have arrived through diverse introduction pathways, either intentionally as described above, or following a clear introduction route from French to northeastern Iberian basins (Clavero and García-Berthou 2006). The remaining non-indigenous vertebrates were evenly native to all the considered regions (Suppl. material 2: fig. S8a), though non-indigenous reptiles were mostly native to North America, as previously documented for Spain (Vilà et al. 2001; Poch et al. 2020). However, this pattern in reptile introductions contrasts with that reported for non-indigenous herpetofauna naturalised in Europe, which was mostly native to Asia and Africa (Kark et al. 2009). Overall, non-indigenous vertebrates were almost exclusively introduced through intentional pathways (i.e. release and escape) (Suppl. material 2: fig. S8b), which is in accordance with continental patterns reported for Europe (Nunes et al. 2015; Saul et al. 2017). Native biogeographic regions for non-indigenous vertebrates correspond to temperate regions with climate regimes similar to the Iberian Peninsula. In this context, NIS introductions from regions with similar climate regimes are more likely to be successful and lead to established NIS (i.e. self-sustaining populations), as these species could be physiologically already adapted to the environmental conditions of the recipient aquatic ecosystems (Ribeiro et al. 2008).

Unlike vertebrates, invertebrate NIS were mostly introduced in Iberian inland waters through two unintentional pathways: contaminant and stowaway. They were native to almost all geographic regions, with North America and temperate Asia being the predominant. Previous studies have shown that most estuarine NIS of non-mollusc and non-arthropod invertebrates (e.g. annelids or platyhelminthes) reached the Iberian coast as hitchhikers through ballast water or hull fouling vessels from global maritime trade (Zorita et al. 2013; Chainho et al. 2015; López and Richter 2017; Cabral et al. 2020). This vector of introduction has been also highlighted as responsible for the arrival of some arthropods (e.g. estuarine crabs) to Iberian transitional waters. Several non-indigenous invertebrates

(e.g. ostracods) have been also passively imported with rice culturing from Asia (Forès 1998; Valls et al. 2014). Non-indigenous decapods were composed by two separate groups, with freshwater crayfish being mostly native to North America and introduced intentionally (i.e. escape or release) for commercial purposes (Vedia and Miranda 2013), whereas estuarine crabs arrived unintentionally through stowaway and were mostly native to America (Muñoz-Mas and García-Berthou 2020). Non-indigenous molluscs arrived to Iberian inland waters mostly through stowaway, contaminants and escape, most of them associated with aquaculture facilities (López-Soriano and Quiñonero-Salgado 2016). The opening and subsequent enlargement of the Suez Canal in 1869 also allowed several estuarine gastropods of Indo-Pacific origin (the so-called Lessepsian migrants) to colonise the Mediterranean Sea (Nunes et al. 2014), and spread over transitional waters of the Iberian coast. For instance, such is the case of the molluscs *Fulvia fragilis*, *Bursatella leachii*, *Pinctada radiata* and *Cerithium scabridum* (López-Soriano et al. 2020). Moreover, anthropogenic modifications of Iberian estuaries may facilitate the establishment of those NIS that are more environmentally tolerant (González-Ortegón and Moreno-Andrés 2021).

The recorded non-indigenous aquatic vascular plants are mainly native to South and North America, and most of them were introduced through escape and release, although a non-negligible number of them also arrived as contaminants. Moreover, up to five different introduction pathways were exclusively associated with the arrival of some non-indigenous aquatic plants (e.g. *Heteranthera limosa*) (Suppl. material 2: fig. S2b). This pattern in non-indigenous plant introduction is congruent with that reported at continental scale in Europe, with escape being the major introduction pathway and vascular plants being also the biotic group introduced through more diverse vectors (Pergl et al. 2017).

Almost half of the recorded non-indigenous macroalgae was native to the Pacific Ocean and considered Lessepsian migrants, whereas the other half was native to the remaining geographic regions and introduced presumably passively through maritime traffic (Chainho et al. 2015; Orlando-Bonaca et al. 2021). Fungi corresponded to pathogens and were exclusively introduced as contaminants, but results provided here for this group are likely biased due to important challenges for taxonomic identification and poor knowledge of their biogeography (Bailey et al. 2020; Turbelin et al. 2022).

Legal coverage and policy implications

Legislative instruments (e.g. regulation lists or catalogues) are developed at European, national and even regional level to prevent the introduction and spread of enlisted NIS through direct management actions. The Regulation (EU) No 1143/2014 established a list of IAS of Union concern which entails that all EU Member States must implement specific management actions to prevent new introductions and further spread across European countries (Genovesi et al. 2015). Spain and Portugal have developed and adapted their IAS legislations to the EU Regulation, with the Spanish IAS catalogue and the Portuguese national IAS list being pivotal for providing legally binding lists that imply a generic prohibition on possession, transport and trade of listed taxa. We found that about 8% of the NIS recorded in Iberian inland waters were included in

the Union list, which is the core of the EU Regulation. On the other hand, 26% and 25% of the recorded NIS were included in the Portuguese and Spanish IAS catalogues respectively, although it is understandable that the national IAS regulations do not necessarily include all the non-indigenous species recorded in their territories. Additionally, almost half (48.5%) of the listed NIS were also listed in the Spanish Allochthonous List, but this regularly upgraded list is focused on taxa potentially introduced in the near future and aims to regulate the importation of new NIS (other than those listed in the Union List and the Spanish IAS List) from other countries that are not part of the European Union and to promote adequate risk assessment. This moderately high percentage is explained by the fact that the Spanish Allochthonous List includes several entire genera (e.g. *Alternanthera* ssp. and *Lepomis* ssp.) and a very large number of species with the aim of regulating the potential importation of allochthonous taxa which are actually sibling species of already introduced NIS (e.g. *Alternanthera sessilis* and *Lepomis gibbosus*) and could lead to similar impacts in Iberian inland waters.

About 17.6% of the listed NIS in the present study have their native range within Europe, thus placing important challenges for transnational regulation and cooperation at Europe scale. This situation may lead to a complex conservation paradox when some aquatic species are native and even threatened in certain EU Member States but they have been introduced and become invasive in others (Marchetti and Engstrom 2016). Hence, national-level regulation instruments must be properly designed and implemented to deal with NIS that are particularly harmful to a given region and address these inherent constraints derived from managing NIS at European scale (Baquero et al. 2023). Consequently, effective management will require that national NIS catalogues are complemented to include all taxa that are considered a priority for management (Angulo et al. 2021). In this context, horizon scan exercises may become particularly useful to identify those high-risk NIS requiring priority management actions within a given region. In fact, Oficialdegui et al. (2023) highlighted a concern list of 126 taxa (all of them included in our inventory making up 38.6% of our listed NIS), as the most relevant invasive alien species already present in Iberian inland waters. Despite the effort made in these kinds of exercises, further research is needed to update the lists, and to address other aquatic invasive taxa that are continually being reported for the first time. The inventory presented here may be useful for this purpose.

On the other hand, our assessment on legal coverage provided by regulation lists highlighted important taxonomic-related biases. For instance, the Union list does not include any non-indigenous mollusc despite most of them being non-indigenous to Europe and some are already causing important ecological and economic impacts in Iberian inland waters (Sampaio and Rodil 2014; Gilioli et al. 2017). Therefore, further European-scale efforts should be done to include non-indigenous molluscs in NIS regulation. Although the Iberian Peninsula can be considered a single biogeographical entity, our results pointed to a considerable mismatch in the criteria followed for species listing between both Spanish and Portuguese catalogues. For instance, several NIS already introduced in both Iberian countries were included in the Spanish IAS catalogue but excluded from the Portuguese one, and vice versa. Hence, independent NIS management

in neighbouring countries belonging to the same biogeographical region can jeopardise resource optimization and ultimately hinder effectiveness of management actions. Supra-national coordinated management actions are particularly needed, as they are generally more effective than national or regional ones (Faulkner et al. 2020). In fact, the Article 11 of the EU Regulation states that coordination and cooperation among MS is pivotal to address a strategic management. Owing to the several river basins shared by Spain and Portugal, the creation of an Iberian office for a coordinated NIS management would likely enhance the effectiveness of control measures and prevention protocols, as well as inter-sectorial communication for improving stakeholder engagement, as already suggested for other regions (Caffrey et al. 2014; Piria et al. 2017). Within the framework of this coordination office, national governments should channel management strategies on aquatic NIS through inter-regional regulation institutions (i.e. river basin authorities and coastal demarcations) to ensure coordinated efforts and avoid constraints from political borders among autonomous communities. The management of NIS is therefore a complex and transnational challenge that requires multi-faceted actions involving diverse institutions and stakeholders at different spatial scales (Baquero et al. 2021). For that purpose, the EU Regulation allows MS to list NIS of regional concern that require enhanced cooperation among involved countries. To inform these NIS management strategies, further research efforts in invasion science should be more applied and focused on cost-efficient actions (Muñoz-Mas et al. 2021).

Conclusions

The introduction of NIS in Iberian inland waters is a long-lasting process affecting many facets of biodiversity, but also local economies and public health. Managing aquatic NIS in the Iberian Peninsula requires a well-coordinated strategy among decision-makers and stakeholders. Nowadays, the increase of human pressure on natural habitats, the climate change and the expanding international trade are promoting the entry, spread, and establishment of new non-native taxa, particularly in inland waters. Hence, effective NIS management requires updated and detailed information on main introduction-related attributes. This study provides a comprehensive multi-taxa inventory of aquatic NIS introduced in Iberian freshwater and transitional waters. This baseline information is delivered through a freely available database intended to become a key tool for improving NIS prevention, monitoring and management at Iberian level. For instance, our assessment may serve as a useful resource for managing NIS introduction pathways into freshwater and estuarine ecosystems, as well as for communicating the magnitude of aquatic invasions to all related authorities and stakeholders. Moreover, this inventory also aims to meet the requirements on updated NIS data stated by the EU Regulation on IAS. Ultimately, our study provides valuable information on the implementation of other EU policies with implications on NIS management, such as the EU Biodiversity Strategy to 2030, the Birds and Habitats Directives, the Marine Strategy Framework Directive, and the Water Framework Directive.

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Conceptualization & Coordinating groups: FJ Oliva-Paterna, JM Zamora-Marín, F Ribeiro, PM Anastácio, B Gallardo, E García-Berthou, P García-Murillo, F Cobo, R Miranda, D Boix, L Medina, F Morcillo, J Oscoz, A Guillén, A Arias, JA Cuesta. Data curation: A Ruiz-Navarro, F Ribeiro, AA Herrero-Reyes, JM Zamora-Marín and FJ Oliva-Paterna. Formal analyses: JM Zamora-Marín, A Ruiz-Navarro, A Guerrero-Gómez and FJ Oliva-Paterna. Writing - Original draft: JM Zamora-Marín, A Ruiz-Navarro, FJ Oliva-Paterna, FJ Oficialdegui. Supervision and Writing - review & editing: FJ Oliva-Paterna, JM Zamora-Marín, FJ Oficialdegui, F Ribeiro, PM Anastácio, B Gallardo, E García-Berthou, P García-Murillo, R Miranda, D Boix, A Arias, JA Cuesta. Investigation, Visualization & Review: All authors. Project Administration: FJ Oliva-Paterna. All authors have reviewed and contributed to improve the text and figures.

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Supplementary material 1

All data of the recorded NIS

Authors: Jose M. Zamora-Marín, Ana Ruiz-Navarro, Francisco J. Oficialdegui, Pedro M. Anastácio, Rafael Miranda, Pablo García-Murillo, Fernando Cobo, Filipe Ribeiro, Belinda Gallardo, Emili García-Berthou, Dani Boix, Leopoldo Medina, Felipe Morcillo, Javier Oscoz, Antonio Guillén, Antonio A. Herrero-Reyes, Francisca C. Aguiar, David Almeida, Andrés Arias, César Ayres, Filipe Banha, Sandra Barca, Idoia Biurrun, M. Pilar Cabezas, Sara Calero, Juan A. Campos, Laura Capdevila-Argüelles, César Capinha, André Carapeto, Frederic Casals, Paula Chainho, Santos Cirujano, Miguel Clavero, Jose A. Cuesta, Vicente Deltoro, João Encarnação, Carlos Fernández-Delgado, Javier Franco, Antonio J. García-Meseguer, Simone Guareschi, Adrián Guerrero-Gómez, Virgilio Hermoso, Celia López-Cañizares, Joaquín López-Soriano, Annie Machordom, Joana Martelo, Andrés Mellado-Díaz, Juan C. Moreno, Rosa Olivo del Amo, J. Carlos Otero, Anabel Perdices, Quim Pou-Rovira, Sergio Quiñonero-Salgado, Argantonio Rodríguez-Merino, Macarena Ros, Enrique Sánchez-Gullón, Marta I. Sánchez, David Sánchez-Fernández, Jorge R. Sánchez-González, Oscar Soriano, M. Alexandra Teodósio, Mar Torralva, Rufino Vieira-Lanero, Antonio Zamora-López, Francisco J. Oliva-Paterna

Data type: xlsx

Explanation note: Excel file containing all data of the recorded NIS, as well as a corresponding legend for an easier interpretation and all the references supporting the NIS inventory.

Link: https://doi.org/10.3897/neobiota.89.105994.suppl1

Supplementary material 2

Supplementary images

Authors: Jose M. Zamora-Marín, Ana Ruiz-Navarro, Francisco J. Oficialdegui, Pedro M. Anastácio, Rafael Miranda, Pablo García-Murillo, Fernando Cobo, Filipe Ribeiro, Belinda Gallardo, Emili García-Berthou, Dani Boix, Leopoldo Medina, Felipe Morcillo, Javier Oscoz, Antonio Guillén, Antonio A. Herrero-Reyes, Francisca C. Aguiar, David Almeida, Andrés Arias, César Ayres, Filipe Banha, Sandra Barca, Idoia Biurrun, M. Pilar Cabezas, Sara Calero, Juan A. Campos, Laura Capdevila-Argüelles, César Capinha, André Carapeto, Frederic Casals, Paula Chainho, Santos Cirujano, Miguel Clavero, Jose A. Cuesta, Vicente Deltoro, João Encarnação, Carlos Fernández-Delgado, Javier Franco, Antonio J. García-Meseguer, Simone Guareschi, Adrián Guerrero-Gómez, Virgilio Hermoso, Celia López-Cañizares, Joaquín López-Soriano, Annie Machordom, Joana Martelo, Andrés Mellado-Díaz, Juan C. Moreno, Rosa Olivo del Amo, J. Carlos Otero, Anabel Perdices, Quim Pou-Rovira, Sergio Quiñonero-Salgado, Argantonio Rodríguez-Merino, Macarena Ros, Enrique Sánchez-Gullón, Marta I. Sánchez, David Sánchez-Fernández, Jorge R. Sánchez-González, Oscar Soriano, M. Alexandra Teodósio, Mar Torralva, Rufino Vieira-Lanero, Antonio Zamora-López, Francisco J. Oliva-Paterna

Data type: docx

Explanation note: **fig. S1**: Number of aquatic non-indigenous species (NIS) recorded in Iberian inland waters (including freshwater and transitional waters) across taxonomic classes; **fig. S2**: Number of native regions (a) and introduction pathways (b) associated to the five major biotic groups containing all aquatic non-indigenous species (NIS) introduced in Iberian inland waters (including freshwater and transitional waters); **fig. S3**: Contribution of the seven categories of introduction pathways to the arrival of aquatic non-indigenous species (NIS) to Iberian inland waters (both freshwater and transitional waters); **fig. S4**: Temporal variation in the overall contribution of the seven introduction pathways to the arrival of aquatic non-indigenous species (NIS) to Iberian inland waters (including freshwater and transitional waters); **fig. S5**: By-country distribution of first European records of aquatic non-indigenous species (NIS) introduced in Iberian inland waters (including freshwater and transitional waters); **fig. S6**: Distribution across functional groups (i.e. trophic groups) of aquatic non-indigenous species (NIS) recorded in Iberian inland waters (including freshwater and transitional waters); **fig. S7**: Legal coverage of official regulation lists for aquatic non-indigenous species (NIS) introduced in Iberian inland waters (including freshwater and transitional waters); **fig. S8**: Distribution of native regions (a) and introduction pathways (b) across taxonomic classes containing all aquatic non-indigenous species (NIS) introduced in Iberian inland waters (including freshwater and transitional waters)

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RESEARCH ARTICLE

Clarification and guidance on the use of the Socio-Economic Impact Classification for Alien Taxa (SEICAT) framework

Anna F. Probert[1,2], Giovanni Vimercati[1], Sabrina Kumschick[3,4], Lara Volery[1], Sven Bacher[1]

1 *Department of Biology, University of Fribourg, Chemin du Musée 15, 1700 Fribourg, Switzerland* **2** *Zoology Discipline, School of Environmental and Rural Science, University of New England, Armidale, NSW, 2351, Australia* **3** *Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Matieland, South Africa* **4** *South African National Biodiversity Institute, Kirstenbosch Research Centre, Cape Town, South Africa*

Corresponding author: Anna F. Probert (afprobert@outlook.com)

Abstract

Understanding the ways in which alien taxa threaten human well-being, beyond purely monetary costs, can be difficult as impacts differ vastly across social, cultural, and economic contexts. Failure to capture impacts outside of monetary costs means that impacts are unfairly weighted towards those that can be easily monetised, which is unlikely to be a realistic measure of how alien species truly affect human well-being. To address this issue, the Socio-Economic Impact Classification for Alien Taxa (SEICAT) was developed with the intention to facilitate standardised classifications and comparisons of the impacts of alien taxa on human well-being and livelihood. The framework measures impacts by assessing to what extent alien taxa have altered human activities, so has application across a broad range of reported impacts associated with different constituents of human well-being. Although in their original paper, Bacher et al. (2018) provided an overview of the SEICAT framework, comprehensive guidelines that enable assessors to overcome potential ambiguities were, until now, unavailable. This may be preventing the broad application of the framework due to reduced usability. Here, we provide clarification and guidance for the application of SEICAT to facilitate standardised, consistent assessments. In particular, we address uncertainties stemming from unclear definitions of impacted communities and activities, as well as the spatial and temporal scales of relevance. We clarify these key issues and give explicit recommendations, whilst encouraging the collection of additional contextual information to be recorded for each assessed impact report, to generate more practical information for end-users of SEICAT data. Further, we recommend

adopting an alternative terminology for the impact categories to reduce the potential misuse of the current descriptors. The intended outcome of this work is to aid the broad application of the SEICAT framework in a consistent and transparent manner.

Introduction

Alien species have the capacity to negatively affect people by impacting various aspects of human well-being and livelihood. For instance, alien plants and insects trigger allergic reactions (Smith et al. 2013; Vidal 2022), alien ungulates cause road accidents (Edwards et al. 2010), alien grasses increase the occurrence and severity of wildfires (Fusco et al. 2019), and alien frogs can affect housing markets (Kaiser and Burnett 2006). Such disparate impacts are inherently linked to the extreme context-dependency in which human communities live, whereby the same alien species can have vastly different impacts depending on the local environmental characteristics and the individual circumstances of people (Global Invasive Species Programme 2006; Tessema 2012; Muller et al. 2017). The ability to characterise and ascribe standardised measures of impact is therefore essential to enable meaningful comparisons because the contexts under which impacts occur, and are observed and reported, greatly differ.

The Socio-Economic Impact Classification for Alien Taxa (SEICAT) framework was developed as a standardised method to classify and categorise the impacts of alien species to human well-being (Bacher et al. 2018). Given well-being is a multi-faceted concept, comprising how people both feel and function (Sen 1993; Cloninger 2004), the framework moves beyond describing impacts in purely monetary terms – which has been a prevailing method of evaluating the effects of biological invasions to socio-economic activities (van Wilgen et al. 2001; Diagne et al. 2021). Central to the SEICAT framework is understanding the way in which alien species affect human activities (Bacher et al. 2018). More specifically, SEICAT conceptualises activities as the "achieved functionings" of the capability approach (Sen 1999; Robeyns 2011; see Bacher et al. 2018), using changes to activities as a proxy to changes to human well-being. In order to capture the ways in which human well-being is affected by alien species, SEICAT links the impact of alien species to constituents of human well-being (i.e. health; security; social, spiritual and cultural relations; material and immaterial assets; adapted from the Millennium Ecosystem Assessment (2005)). These can then be connected to the way in which alien species impact socio-economic dimensions of human well-being. By identifying the relevant constituents of well-being affected, a broad understanding of how alien species affect peoples' capabilities is obtained. Using this approach enables greater recognition of how alien species can differentially affect people within similar and dissimilar socio-economic contexts. This is because some individuals, or wider communities, will have the means to compensate or tolerate

impacts more so than others due to various reasons including the inequalities and inequities associated with resource distribution, access to opportunities and personal circumstances. For instance, the potential loss of locally performed recreational activities may be compensated for by travelling farther at additional costs – in both money and time – that not all individuals in all communities will be able to afford.

SEICAT identifies how, and to what degree, activities performed by humans are affected by alien taxa. This allows impacts across all different social, cultural, and economic contexts to be assigned one of five semi-quantitative categories of severity based on the extent to which the alien taxon impacts the individual- and community-level way of life (Table 1). The framework emphasises that not all activities will be valued equally by different people and the intended outcome of SEICAT assessments is to identify consequences in a standardised manner rather than produce a prescribed weighted summary of impacts (Bacher et al. 2018). Impact data are obtained by conducting a review of the scientific and non-scientific literature targeted towards the focal alien species. These data may come from a range of different sources such as observational or experimental studies from the ecological, medical, and/or social sciences, government reports and media interviews. Consequently, the impact data can be collected and communicated in different ways meaning that information must be critically assessed for its relevance to the SEICAT criteria. Once a literature search is complete, an assessor reads each document to identify reported impacts to include in the assessment; within each document there may be several impacts (from one or more alien taxa) reported of relevance to the SEICAT criteria, or indeed, none. Each relevant impact is then linked to the relevant constituent(s) of well-being and assigned a confidence score of either low, medium or high, to indicate the level of certainty that the given impact classification represents the real situation – an important component of the assessment process given impacts can derive from quantitative or qualitative data of differing quality that also vary in terms of scope and scale (Probert et al. 2020). SEICAT thus aims to provide a method of assigning impacts across different contexts where alien species have been introduced based on the available evidence.

Although the original SEICAT publication by Bacher et al. (2018) provided a conceptual basis for the application of the framework, specific guidance that addresses a clear and nuanced understanding of the criteria is currently lacking. This is of particular importance given the recent advocacy for using the framework by the Convention on Biological Diversity (CBD 2023). Given that impact data are derived from different sources and not communicated with the SEICAT criteria in mind, an unambiguous understanding of how assessors should translate impacts into one of the five categories is necessary to ensure consistency in scoring among assessments. Consistent application among assessors is a major challenge for impact scoring systems (González-Moreno et al. 2019; Clarke et al. 2021; Bernardo-Madrid et al. 2022) and a clear conceptual basis that permits congruous scoring is crucial. In order to better fulfil the intention of SEICAT, conceptual and practical ambiguities that currently persist must be addressed. Indeed, new frameworks and tools within invasion science should be modified and adapted as additional knowledge is acquired, allowing future iterations

Table 1. Overview of the five semi-quantitative impact categories of the SEICAT framework. Impact categories for preferred activities follow those proposed by Bacher et al. (2018). New additions recommended in this manuscript, including a change to impact category terminology, are preceded by an asterisk. Note that when there is no or inadequate information to classify an alien taxon to one of the five SEICAT impact categories, the taxon should be classified as Data Deficient (DD).

Impact category	Impact criteria	
Terminology	For preferred activities (after Bacher et al. 2018)	*For non-preferred, burdensome activities
Minimal Concern (MC) *Category 1 (C1)	No deleterious impacts reported despite availability of relevant studies with regard to its impact on human well-being.	*No change in any existent, non-preferred, burdensome activity compared to the scenario in which the alien species was absent.
Minor (MN) *Category 2 (C2)	Negative effect on peoples' well-being, such that the alien taxon makes it difficult for people to participate in their normal activities. Individual people in an activity suffer in at least one constituent of well-being (i.e. security; material and non-material assets; health; social, spiritual and cultural relations). Reductions of well-being can be detected through e.g. income loss, health problems, higher effort or expenses to participate in activities, increased difficulty in accessing goods, disruption of social activities, induction of fear, but no change in activity size is reported, i.e. the number of people participating in that activity remains the same. Also includes scenarios where novel activities commence as compensatory measures of an alien species impact (see right column).	*Existent, non-preferred, burdensome activities increase in frequency and/or intensity because of the alien species, but no change in activity size is reported, i.e. the number of people participating in the burdensome activity remains the same.
Moderate (MO) *Category 3 (C3)	Negative effects on well-being leading to changes in activity size, fewer people participating in an activity, but the activity is still carried out. Reductions in activity size can be due to various reasons, e.g. moving the activity to regions without the alien taxon or to other parts of the area less invaded by the alien taxon; partial abandonment of an activity without replacement by other activities; or switch to other activities while staying in the same area invaded by the alien taxon. Also, spatial displacement, abandonment or switch of activities does not increase human well-being compared to levels before the alien taxon invaded the region (no increase in opportunities due to the alien taxon).	*An increase in activity size for an existent, non-preferred, burdensome activity is reported because of the alien species, i.e. the number of people involved in the burdensome activity increases.
Major (MR) *Category 4a (C4a)	Local disappearance of an activity from all or part of the area invaded by the alien taxon. Collapse of the specific social activity, switch to other activities, or abandonment of activity without replacement, or emigration from the region. Change is likely to be reversible within a decade after removal or control of the alien taxon. "Local disappearance" does not necessarily imply the disappearance of activities from the entire region assessed, but refers to the typical spatial scale over which social communities in the region are characterised (e.g. a human settlement).	*People commence a novel non-preferred, burdensome activity – that was previously not performed before the alien species was present – because of the alien species. Such an activity would cease upon the hypothetical removal (or control) of the alien species.
Massive (MV) *Category 4b (C4b)	Local disappearance of an activity from all or part of the area invaded by the alien taxon. Collapse of the specific social activity, switch to other activities, or abandonment of activity without replacement, or emigration from the region. Change is likely to be reversible within a decade after removal or control of the alien taxon. "Local disappearance" does not necessarily imply the disappearance of activities from the entire region assessed, but refers to the typical spatial scale over which social communities in the region are characterised (e.g. a human settlement).	*People commence a novel non-preferred, burdensome activity – that was previously not performed before the alien species was present – because of the alien species. Such an activity would continue upon the hypothetical removal (or control) of the alien species.

to improve their application. In this paper, we aim to clarify potential sources of uncertainty related to 1) the definition and scoring of different preferred and burdensome activities, 2) the definition of activity size (that is the community of interest for which we are categorising impacts), and 3) the spatial and temporal scales of relevance for impact reports (see Box 1). We provide thorough explanations to reduce inconsistencies among assessments and improve comparisons of impact data collated using SEICAT. Further, we highlight that the current impact category terminology (Minimal Con-

Box 1. Summary of the main guidance and recommendations outlined in this paper.

1. SEICAT can ascribe impacts to both beings and doings yet impacts to the former will always be limited to scores of Minor*. This has important consequences when assigning confidence scores and interpreting SEICAT data. *except in cases where the alien species causes mortality, which is always scored at least as Moderate.

2. Non-preferred, burdensome activities are relevant to SEICAT and should be scored according to the new criteria proposed.

3. Constituents of well-being that are affected by alien species are not mutually exclusive and often must be inferred by assessors. Additional explanations should be provided by assessors to illustrate when constituents of well-being are provided within an impact report versus inferred by an assessor.

4. Impacted activities will be described at different specificities across impact reports. This has significant implications when interpreting SEICAT data. Assessors can generate increasingly transparent and practical assessment data by factoring in the hierarchical nature of activities.

5. Accurate evaluation of impacts requires knowledge of activity size (i.e. the number of individuals who performed the activity prior to the arrival, or impact, of the alien species). As we are in essence interested in people, rather than activities, assessors need to account for the fact that changes in activity size may not be reported in a way in which individual identity is clear.

6. Impacts should not be linked to specific areas as this does not account for our community of interest. Rather, the focus should be on a group of people as this accounts for when people decide to conduct their usual activities elsewhere in response to an alien species.

7. Impacts are subject to temporal change and depending on the timeframe in which they are reported, the impact score may be under- or overestimated. Assessors should be aware of the differences between ephemeral and longer-term impacts.

8. Adopting more neutral language for describing the category of impacts could help to reduce the potential misuse and misinterpretation of SEICAT data.

cern, Minor, Moderate, Major, Massive) may act on assessors' subjective judgement and, moreover, has the potential to be misinterpreted and misused to suit agendas of key stakeholders in species management. To avoid this, future assessments should adopt more neutral terminology to describe categories of impact magnitude. Lastly, we provide a template for data recording (see Suppl. material 1), recommending that assessors record additional information related to each impact report, to generate more useful socio-economic impact databases for end-users and facilitate future analyses to better understand how context relates to impact magnitude.

SEICAT and the capability approach: translating reported impacts into impact scores

The SEICAT framework was developed based on the capability approach to ascribe understanding of alien species impacts to human well-being (Sen 1979, 1999). Under the capability approach, well-being is conceptualised as functionings, where the focus is on what individuals are able to do and be, and capabilities, which reflect the actual opportunity individuals have to achieve these functionings. This allows well-being to be considered for individuals in terms of both what people are *able* to do and what they *choose* to do. Importantly, the capability approach focuses on the end outcomes for people, which means an individual's specific circumstances can be better incorporated into the concept of well-being.

In order to classify changes to activities, it is necessary to understand what activities are. Yet, understanding what constitutes an activity may be causing confusion, and thus inconsistencies, among assessors given that SEICAT also permits impacts that are not measured through changes in activities to be scored (Fig. 1).

Bacher et al. (2018) define an activity as "any human endeavour that is, or could be, affected in its entirety by an alien taxon". This aligns with the capability approach where an endeavour can be considered as both the doings and beings of people, wherein doings are actual activities (e.g. swimming, gardening, farming) and beings are states that a person has achieved (e.g. being healthy, being educated, being rested, being financially stable). Thus, SEICAT permits not only using changes to human activities as indicators of impact but also other changes to various dimensions of wellness, for example, reports of individuals feeling less happy or healthy because of an alien species. The argument proposed for the inclusion of such impacts by Bacher et al. (2018) can be based on the assumption that when changes to people's beings become significant enough to cause a person to suffer, in many cases, they may translate into changes to their activities (Fig. 1).

The inclusion of impact reports detailing changes to peoples' states of being represents a potential ambiguity that requires further clarification. Although Bacher et al. (2018) alluded to the inclusion of beings as relevant to SEICAT, there was no explicit guidance
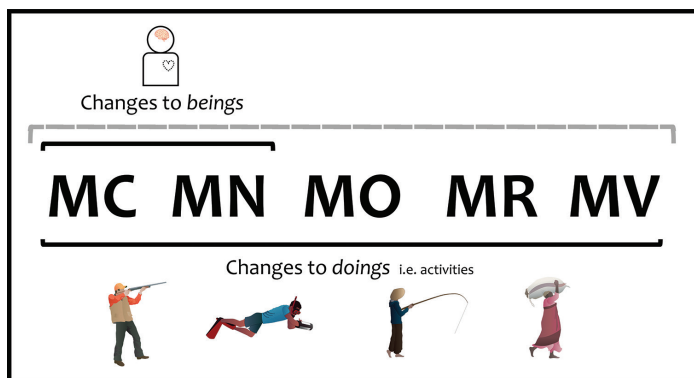
Figure 1. The five different impact scores that can be classified using SEICAT to assess the impacts of alien species to aspects of well-being in terms of what people do (doings) and how they feel (beings). Impact reports that mention changes to beings can only be assigned an impact score of Minor at the highest, even though the real impact might be higher (this uncertainty is captured in a lower confidence score). For instance, alien species may affect people's health where impacts are reported as people feeling less physically or mentally well as a result of an alien species. Other examples include impact reports stating individuals requiring medical advice or treatment as the result of an alien species would also be considered as impacts to beings. This does not necessarily mean that the alien species does not alter the activities of people, indicated by the grey dashed bracket, however, these impacts cannot be assigned a higher score as impact scores above Minor require information on peoples' activities (doings) in relation to the alien species. In most cases, changes to beings will result in changes to people's activities to some degree (e.g. by making them less-enjoyable or more difficult to perform) but often such information is not reported. Symbols obtained from the Integration and Application Network (ian.umces.edu/media-library).

of how such impact reports should be included in assessments. Conceptually, impacts of alien species reported in a way that link to either doings or beings can be scored but a critical difference between the two must be realised by assessors: any reported impact affecting a being (Table 2) – in the absence of any supporting information about how this translates to a change in activity – should be limited to a maximum score of Minor. This is because impacts above Minor, by definition, require information about whether individuals stop performing activities (Table 1, Fig. 1). In such cases where an impact to a being is scored, and it is unknown from the impact report whether – and to which extent – activities are affected, low confidence should accompany the impact score given the uncertainty whether or not the alien species is in actuality causing changes to peoples' activities (Fig. 1).

It should be noted that an impact of an alien species can, therefore, derive a score of Minor based on two differing scenarios: i) an observation where there was no evidence that any individuals abandon an activity due to an alien, although there was evidence that the alien species altered the duration or frequency at which the activity is performed, thus resulting in decreased well-being (Table 1) or, as discussed in the paragraph above, ii) an observation where there was evidence that an alien species negatively affected the mental or physical state of individuals' beings with unknown or unreported consequences for changes to activities. These two scenarios represent quite different impact situations that can be reflected in their confidence scores. For instance, the former can be assigned different levels of uncertainty depending on various aspects such as data quality, temporal and spatial scale, etc. (see Probert et al. 2020). In contrast, the latter scenario will likely always be assigned low confidence as the scope of the reported impact does not account for activities and, therefore, it cannot be determined based on the available information if activities are being affected. Differentiation between whether the impact report derives from scenario i) or ii) is, therefore, useful to ensure that SEICAT data is interpreted appropriately by end-users. A practical distinction can easily be made during the assessment procedure by including an additional column containing this information (see Suppl. material 1).

The common reporting of impacts that link the impacts of alien species to beings rather than doings is one of the reasons why there are many impacts assigned Minor with low confidence (e.g. Galanidi et al. 2018; Kenser and Kumschick 2018; Evans et al. 2020; Gruber et al. 2022; Jansen and Kumschick 2022; see Suppl. material 1: worksheet C).

Relevance of non-preferred, burdensome activities and novel, preferred activities to SEICAT

An important consideration of how alien species impact human well-being is that in some cases, people mount a compensatory response to mitigate their negative effects, specifically by expanding existent, or initiating new, non-preferred activities. Such activities can be considered as 'burdensome activities', and their assessment was not explicitly accounted for in Bacher et al. (2018). Since the foundational basis for SEICAT was that any change to an activity caused by an alien taxon would reflect some degree

Table 2. Examples taken from the literature where Minor impacts are scored for impact reports that detail an alien species affecting beings of individuals, rather than activities (doings). By definition, most impacts that are only reported at the level of beings cannot be assigned impacts higher than Minor as these are measured by changes to activities, that is the doings in the capability approach. The one exception to this is when mortality is recorded, in which cases, at least Moderate is always assigned. Scoring impact reports of beings using SEICAT provides important information on how alien species can affect different aspects of human livelihood and well-being and likely translate to changes in activities.

Alien species and country of introduction where impact was recorded	Quotation	Outcome	Constituent of well-being affected	Reference
Silver wattle (*Acacia dealbata*) introduced to South Africa	A few respondents at each site stated that they did not want *A. dealbata* anywhere near their villages because it might harbour criminals. They stated that the presence of *A. dealbata* allowed criminals to hide which would endanger the community. The majority of the respondents stated that the current abundance levels attracted a lot of criminals to their areas. In Caba village near Matatiele, several households had experienced theft of their livestock and they implicated *A. dealbata* because, they argued, it provides cover in which thieves hide and monitor the activities of the residents. Women also expressed fears of going to collect firewood as criminals would hide in *A. dealbata* patches.	Causing fear for safety (being afraid)	Safety	Ngorima and Shackleton (2019)
Wild dog (*Canis lupis familiaris*) introduced to Australia	There can be significant emotional upset and frustration associated with a wild dog or dingo attack on farm stock. Farmers spoke of 'the emotional upset of seeing animals hurt', 'gut wrenching' attacks and 'strong feelings of revenge and contest'.	Causing emotional upset (being emotionally upset)	Social, spiritual and cultural relations; material and immaterial assets	Fitzgerald and Wilkinson (2009)
Wild dog (*Canis lupis familiaris*) introduced to Australia	There is also a sense of psychological insecurity and uncertainty that farmers live with on a daily basis when wild dogs are present in the environment: 'One is always anticipating the possibility of wild dog attack. Whenever one goes into a sheep paddock one thinks "am I going to find a dead sheep here?".'…Farmers also experience a degree of anxiety and uncertainty over their rights with respect to reducing the risks from wild dogs and other pest animals.	Causing anxiety (being anxious)	Social, spiritual and cultural relations; material and immaterial assets	Fitzgerald and Wilkinson (2009)
Montserrat whistling frog (*Eleutherodactylus johnstonei*) introduced to Brazil	In São Paulo, Brazil, a citizen of the invaded neighbourhood in Brooklin has reported a disorder related to chronic stress due to the noise produced by *E. johnstonei*. This disorder eventually caused her to be hospitalised.	Causing chronic stress (being stressed)	Health	Melo et al. (2014)
Rose-ringed parakeet (*Psittacula krameri*) introduced to Hawai'i, USA	On Kaua'i, property owners of apartments, condominiums, and hotels complain about the noise from [Rose-ringed parakeet] ..[]. Similar complaints have been voiced on O'ahu, particularly from apartment residents adjacent to the largest RRP evening roost on O'ahu that is a large *Ficus* sp. tree on Beretania and Punahou Streets (A.B.S. and N.P.K., pers. obs.).	Causing noise disturbance (not being at peace)	Health	Shiels and Kalodimos (2019)
Brown marmorated stink bug (*Halyomorpha halys*) introduced to Maryland, USA	The unpleasant odour emitted when brown marmorated stink bugs are disturbed, and for which they are named, was far less unpleasant than the perceived nuisance caused by their sheer numbers and daily presence. For the period 1 January 2011 through 31 May 2011, on 56% of days 25 or more stink bugs were collected on the first and second floors, and 100 or more were collected on 21% of days.	Causing nuisance through odour and sheer abundance (not being at peace)	Health	Inkley (2012)

of suffering to individuals, scoring criteria focused only on changes to preferred activities that were already being performed. This was based on the implicit assumption that only changes to existing preferred activities are to be assessed, then conflating all impacts on non-preferred activities to Minor impacts. Indeed, Bacher et al. (2018) stated that, among others, Minor impacts are "…Reductions of well-being [that] can be detected through e.g. income loss, health problems, higher effort or expenses to participate in activities…". However, the existing literature often reports cases in which people do not alter their preferred activities (e.g. farming a crop) as a response to alien species (e.g. a crop pest), but rather they initiate or undertake compensatory activities, such as management activities, to secure and maintain well-being. A more explicit consideration of these activities in SEICAT might expand its functionality, as well as its applicability to a broader range of impact scenarios.

Examples of compensatory non-preferred activities include when farmers have to reinforce pest control activities (Dent and Binks 2020), or when boat owners have to initiate maintenance activities – previously unnecessary – to remove and prevent biofouling (Peters et al. 2019), both in response to an alien species. Whilst often related to alien species management, not all burdensome activities will be related to control or prevention. For instance, the unwanted presence of alien species at localities where people usually, and preferentially, perform certain activities may mean they now have to travel (travel being the new activity that is a burden) to new localities where the alien species is absent. In order to account for such impacts found during SEICAT assessments, the current guidance for scoring needs to be expanded. A rational suggestion would be to consider such non-preferred activities among the activities relevant for assessment, so that their initiation or reinforcement can be used as a proxy for negative impacts on well-being.

Analogous to the classification of preferred activities, but with opposite direction, we suggest classification of burdensome activities in five steps as: (Minimal Concern) no change in burdensome activities compared to without the alien species; (Minor) burdensome activities increase in frequency or intensity, but no increase in number of people participating in burdensome activities; (Moderate) increase in number of people involved in burdensome activities; (Major/Massive) initiation of burdensome activities that were formerly not performed, that can be abandoned after hypothetical removal of the alien species or will need to continue, respectively (Table 1).

Consider a hypothetical scenario in which biofouling of an alien mussel species causes damage to boats used for recreational fishing. This situation necessitates the adoption of vessel cleaning – which is a non-preferred activity – in order to counteract the adverse effects of mussels on human well-being (see Fig. 2). If the non-preferred activity was already conducted prior to the focal alien taxon arrival (for instance to control other already present biofouling agents) and no change has been reported, this impact would be classified as of Minimal Concern. If the cleaning was already implemented but changes to this activity occur because of the alien species, for instance cleaning now takes longer and/or must be conducted more frequently, or the number of people performing cleaning increases, such impacts would be considered as Minor or Moderate, respectively. If the non-preferred activity had to be initiated in response to alien mussels,
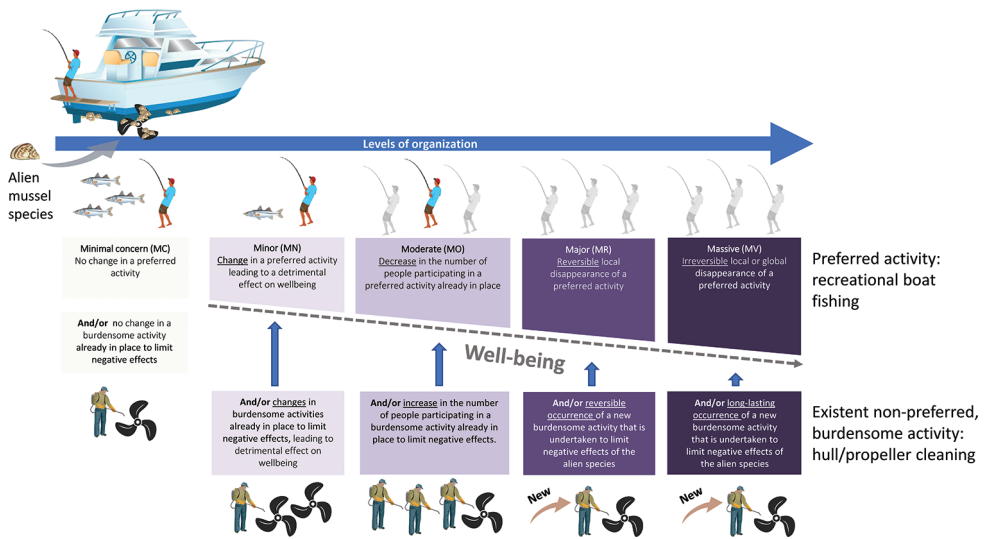
Figure 2. Scoring of SEICAT categories, demonstrating that an alien species can reduce well-being by affecting both preferred and non-preferred, burdensome activities. Here, descriptions for each scoring category are shown with illustrations for different scenarios where an alien mussel species could hypothetically affect the preferred activity of recreational boat fishing and also the non-preferred, burdensome activity, which is the cleaning of the hull and propellers of the boat. Symbols obtained from the Integration and Application Network (ian.umces.edu/media-library).

and is an entirely novel activity for all people in the community of interest, an impact of Major or Massive would be assigned in accordance with their reversibility (see Table 1).

It is important to note that non-preferred activities do not belong to the capability set, i.e. the opportunity set of potential activities (Bacher et al. 2018). In fact, non-preferred activities are generally undertaken in an effort to prevent alien species from negatively affecting preferred activities. Considering one of the examples provided above and illustrated in Fig. 2, boat hull cleaning (the non-preferred activity) is undertaken to mitigate the negative effects that alien mussels have on recreational boat fishing (the preferred activity). While preferred activities can be undertaken regardless of burdensome activities, the latter are pursued only to prevent or mitigate negative consequences of alien species on preferred activities or on the environment. Distinction between changes in preferred and non-preferred activities is therefore critical to meaningfully interpret SEICAT assessments and we emphasise that these two types of activities are kept separate while assessing impacts (see Suppl. material 1: worksheet B). For instance, if an alien species that causes a decline in the number of people involved in recreational boat fishing also provokes the initiation of a new burdensome activity such as hull cleaning (see Fig. 2), this impact should be classified as Moderate for the preferred activity and Major for the burdensome activity, with whether the impact relates to preferred or burdensome activities identified in an additional column. Keeping preferred and burdensome activities separate avoids double counting of impacts when summarising SEICAT data for end-users, as it is expected that the time allocated to bur-

densome activities (performed to mitigate the impact of alien species) will encroach on the time allocated to preferred activities. A critical interpretation of the different meanings of burdens and preferred activities will help to reduce introducing potential biases.

A further point worth clarifying is that there are some cases where impact reports are not relevant to SEICAT assessments, for instance, when alien species create new opportunities (i.e. preferred activities) thereby increasing the capability set of people. In these situations, alien species are considered as beneficial to people which is not relevant under SEICAT. Although measuring and quantifying the positive/beneficial impacts of alien species certainly warrants more attention to improve our understanding of impacts and aid prioritisation (Vimercati et al. 2020), positive impacts are not captured by the SEICAT framework and should not be considered in SEICAT assessments.

Constituents of well-being

The SEICAT framework assigns one or more of the four core constituents of well-being (health; security; social, spiritual and cultural relations; material and immaterial assets) to each reported impact. Each of these constituents is fundamental to the overarching constituent of freedom of choice and action, which is intrinsically linked to the opportunity to be able to pursue and obtain what people value being and doing.

The impacts of alien species to socio-economic dimensions of human well-being and livelihood are highly context dependent since people live in different environmental, socio-political and economic settings but also because individuals can have different motivations for performing the same activity. Linking constituents of well-being to each impact can help highlight these differences, providing different contexts in which impacts are occurring. For example, the effects of an alien pest species that causes significant damage to crops (leading to a reduction in agricultural activities) may result in different consequences for people that farm for subsistence versus those that commercially farm. It may be that for the commercial farmer, loss of income due to crop failure best links to material and immaterial assets whereas for the subsistence farmer, this impact may also link to health in that crop failure leads to a deficiency in obtaining adequate nourishment. This example also demonstrates how constituents of well-being for peoples' impacts are not mutually exclusive. The crops of the subsistence farmer are still a material asset and the activity of farming is often related to social and cultural relations via traditional practices that can be negatively impacted when disrupted by alien pest species.

How impacts relate to constituents of well-being is sometimes clearly stated in an impact report. For example, the invasion of the alien tree *Acacia dealbata* in rural villages of Eastern Cape, South Africa, has induced fear of attack among women who collect firewood due to the trees providing patches for criminals to conceal themselves (Ngorima and Shackleton 2019). Here, the constituent of well-being being affected can directly be discerned as safety. However, oftentimes assessors will have to infer which constituent(s) of well-being are most appropriate for an impact. To provide better context, thus facilitating more meaningful interpretations of SEICAT data, assessors may want to identify where constituents are inferred versus when they are more clearly indicated in the text.

The hierarchical nature of activities – at what level should an activity be assessed?

The human activities that are affected by alien species can be defined and reported at different levels of specificity. This is of great importance for assessors to recognise and understand given the implication for scoring impacts. Activity specificity represents a notable challenge in ensuring that the SEICAT framework standardises impacts in a manner that allows meaningful comparisons because depending on the specificity at which an activity is assessed, the appropriate impact score can be markedly different. Much of an assessor's ability to assign an impact score will depend on the specificity and context under which an impact is reported. Here, issues arise because if activities are too specific and no broader context is available, impacts will not be consistently scored.

To illustrate this point, take a hypothetical example of an impact report that details a complete cessation of swimming in a lake because of an alien species (see Fig. 3A, B). For the purpose of the example, we will assume there are no other lakes in reasonable proximity, meaning people cannot swim elsewhere. If the focal activity for an assessment is considered as "swimming", the most appropriate impact score would be Major or Massive depending on reversibility (see Table 1). However, it is unlikely that swimming is the only activity performed in or on the lake and it could be that whilst all activities where people are fully, or partially, submerged in water have to be abandoned because of the alien species, other activities, such as kayaking and sailing, are carried out as usual (and thus for these specific activities the impact score would be Minimal Concern). It could be that an impact report rather notes a reduction in "water sports" as opposed to more specific water-based activities. The activity water sports would in actuality consist of several activities that are differentially affected by the alien species, such as swimming and sailing (swimming has to be abandoned but sailing can continue per usual), with no further specificity about individual activities. In this case, if the focal activity for an assessment is considered as "water sports", as fewer people participate (all swimmers abandoned swimming) the most appropriate impact score would be Moderate (see Table 1).

To potentially overcome this issue, Bacher et al. (2018) suggest to aggregate activities at the largest activity that could possibly be affected as a whole – thus, for the example above, the activity "water sports" would be scored. This does require knowledge about the alien species and the nature of its impacts that may not necessarily be contained within impact reports. Arguably, determining what constitutes the largest activity that could be affected is not straightforward and relies on assessors using subjective judgement which introduces a potential form of bias (see Probert et al. 2020) that can reduce the standardisation of impacts across taxa. Further, assessors aggregating impacts this way may unnecessarily lose important details and context that are of use to SEICAT data end-users. For these reasons, we suggest assessors acknowledge the hierarchical nature of activities and include different levels of activity specificity when possible (Fig. 3). Taking this approach means that assessors should score at the level the impact is reported but also consider how this can fit into a hierarchy of activities, particularly in context of the entire assessment. This is because in many cases, similar impacts will be reported for (functionally) similar alien taxa as they often affect people
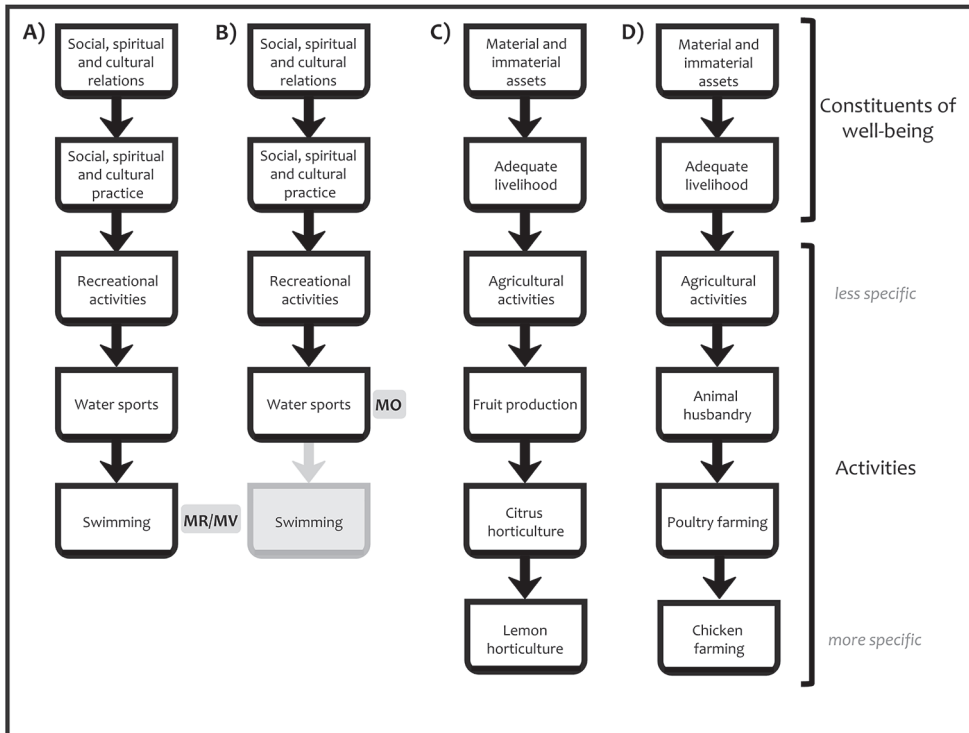
Figure 3. Schematic demonstrating how the different constituents of well-being (under SEICAT, material and immaterial assets; subcategory adequate livelihood) can link to activities that can be defined hierarchically. In the examples **A** and **B** an alien species renders a lake unsuitable for any activity where people are submerged in water (e.g. because the alien species has toxic or skin irritating properties). For **A** the lowest activity being scored is "swimming" and a score or Major/Massive (MR/MV) is assigned as all people abandon this activity because of the alien. In **B** the same scenario is being assessed but the lowest activity being scored is "water sports". Although all swimmers abandon the activity of swimming, some people continue with other activities on the lake such as kayaking and sailing, thus for the activity "water sports" a score of Moderate (MO) is assigned. This demonstrates how the level, or specificity, at which impacts are reported can result in different impact scores. The ability to be able to assign scores will be based on the level of information that is available to an assessor. In **C** and **D** examples of different levels of activities that may be under the umbrella term "agricultural activities" are shown. It is possible that other higher- or lower-level of specificity of activities could be defined but assessors should consider levels that are most of use for comparisons in their assessments. Future assessments should consider activity specificity when applying impact scores and may benefit from ascribing scores in hierarchical natures as illustrated in this diagram. SEICAT data will be more useful and informative if the relevant levels are considered and included within a single assessment.

through similar ways. For example, invasive aquatic plants tend to smother freshwater ecosystems, meaning activities performed in and on lakes are usually directly affected.

By scoring activities at different specificities, end-users of SEICAT data should be better equipped with the necessary information to standardise impacts based on their needs if these data are incorporated into the assessment spreadsheet (provided as Suppl. material 1). Doing so will allow information at different activity levels to be accounted

for in downstream analyses. Whilst for global comparisons and summaries of impacts, it is likely that the highest impact recorded would be most relevant depending on activity specificity, for local decision-makers and stakeholders, the different levels of activities and their impact scores will likely be more important and provide crucial context.

Defining activity size – who is the community of interest?

One of the core tenets of SEICAT is that the magnitude of an impact is measured by the effect on changes in human activity. In particular, to be able to assign an impact score of Moderate or above, information must be available indicating that the number of people participating in an activity (the definition of "activity size" in Bacher et al. 2018) is reduced because of the alien species. However, understanding the concept of activity size can be ambiguous, particularly in light of the differences in impact reporting and the nature of different activities.

A clearer distinction is required to clarify the concept of activity size to reduce the potential ambiguity that may lead assessors to interpret the same information differently. From a conceptual standpoint, activity size should be considered as all the people in the community of interest participating in an activity *before* the alien species caused impacts. Therefore, to accurately determine changes in activity size we would require information regarding the individual identity of people within the community of interest and their personal response (i.e. change in activity) to the arrival (or perceived impact) of the alien species (Fig. 4).

Using only the total number of people participating in an activity – and how it changes in response to an alien species – without any reference to their individual identity, centres the impacts to the activity rather than to the people affected. This then means that the true impacts of alien species to facets of human well-being are not being captured. For instance, there may be cases whereby some people stop performing an activity completely because of an alien species, but others take up the activity (despite or due to the alien species), resulting in no measurable net reduction in the total number of people that perform the activity (Fig. 4). Scoring this as no change in activity size would ignore the evident impact on those people that stopped their activity in response to the alien. People themselves are thus not replaceable and assessors must bear this in mind when assigning impact scores. Although it is plausible that for some people the presence of an alien species could lead to the uptake of new preferred activities, such beneficial impacts are not of relevance to a SEICAT assessment (see section above on novel preferred activities and Fig. 4).

In practice, this information is often not available within impact reports; people's identity is usually unknown except perhaps in situations where data are derived from questionnaires. However, to account for this uncertainty lower confidence may be assigned where appropriate to indicate that the true impact score could be different from the one reported.

Defining the community of interest – that is the specific group of people whose activities are affected by an alien species – can be of central importance to capture flow-on
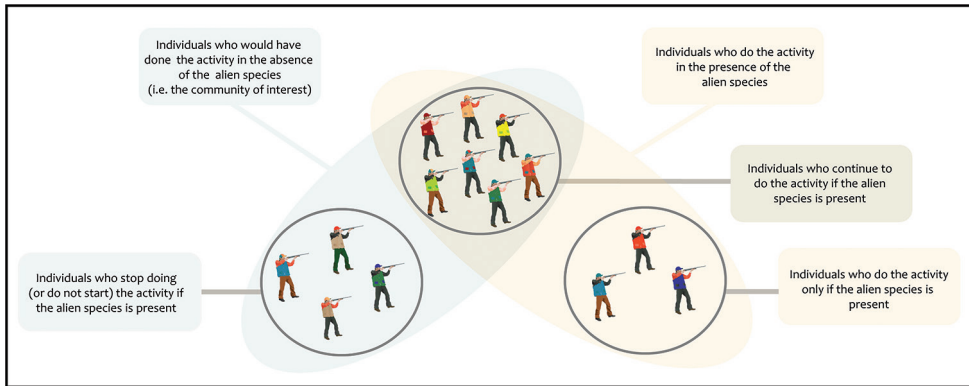
Figure 4. Identity of people performing the activity is important to define activity size. Each hunter here represents a different individual within a group of people that either perform the activity in the presence (yellow oval) or absence (blue oval) of an alien species (e.g. a deer). In this example, the activity is generalised as hunting i.e. it is not defined as hunting a specific species. Note the overlap between the two ovals indicating that the two situations are not mutually exclusive; an individual may perform the activity irrespective of whether the alien species is present or not such that when the alien deer is not present, the individuals continue to hunt albeit a different species. Here, some individual hunters stop hunting once the alien has been introduced (perhaps because it has largely replaced their favourite game species), while other individuals take up hunting because of the alien species. Although in this case more people are hunting in presence of the alien than in its absence, this example would still be considered as a Moderate impact in SEICAT (decrease in the activity size), because people stop the activity because of the alien. The fact that other people pick up the activity due to the alien presence is not considered in SEICAT as only individuals that were participating in the activity prior to the alien species arrival are of interest. Concretely, the community of interest for this example are the 11 individuals in the large grey oval; since four abandon the activity due to the alien, we have evidence that fewer individuals are participating in the activity and therefore can justify the appropriate impact category of Moderate. Symbol obtained from the Integration and Application Network (ian.umces.edu/media-library).

impacts where an alien species' impacts on one group of people subsequently affects other individuals. For example, a reduction in a specific crop caused by an alien pest species can impact both commercial growers, by reducing their income, and consumers that rely on that crop for sustenance. Recognising that growers and consumers represent two distinct communities of interest affected by the same alien species may enable us to better disentangle chain-effects and unravel the complexity of socio-economic impacts.

Understanding the relevant spatial and temporal scales

Assessors should be aware that activities should not be defined in relation to space or time, however, it is important to understand that these two aspects are relevant to how we measure impacts. Understanding the spatial and temporal scales is particularly informative when evaluating the degree of confidence assigned to an impact score.

The spatial scale of an impact measurement

Impact measurements for alien taxa are subject to considerable context-dependency when viewed at different spatial scales (Essl et al. 2017). SEICAT assessors must be able to determine the relevant spatial scale at which an impact score should be assigned. However, this can be challenging given that impact reports will invariably contain different spatial scales and encompass communities of different sizes.

The relevant spatial scale at which impacts should be assessed must consider the 'community of interest'; that is, the group of individuals participating in an activity that *can* be affected by an alien taxon, and are relevant to measuring changes in activity size (see section above). The distinction of *can* is necessary to ensure that assessors are aware that the community of interest may be a subset of people within a surveyed community, i.e. the surveyed community is not necessarily representative of the community of interest. Making this distinction can be difficult, however, given the complexities of human behaviour. For example, if households within a hypothetical town were surveyed to determine if an invasive alien fire ant was affecting their gardening activities it may be reasonable to conclude that the community of interest would comprise only of those households who have the fire ant occurring on their property, and therefore the community of interest directly overlaps with the range of the alien species. However, it is also possible that some individuals beyond the range of the alien species alter their activities out of fear that the fire ant is present (when it is not) or might be in the future.

To illustrate simply why understanding the community of interest is important for impact scoring, take the above scenario, where fire ants affect some households in a suburb, and assume that only people who have the fire ant on their property change their activities. If the town's population was 2000 people but only 30 people lived in properties affected by the fire ant, and all those people had to completely stop gardening due to the infestation, then the level at which we focus the community of interest is important to scoring. If our community of interest is the entire town, the score would be Moderate, whereas if we only include those that have the fire ant present on their property, the score would be Major or Massive depending on whether the fire ant could be controlled and the impact reversed. Being able to discern this, will likely be dependent on the information available in the impact report. Any uncertainty an assessor has regarding whether the impact report accurately reflects what is truly happening can be reflected by lowering confidence.

Assessors should be aware that in some circumstances communities of interest can be situated at great distances from where the focal alien species is established. For example, an alien species affecting water quality of a river or other water body could hypothetically have significant impacts on communities who rely on that water many kilometres downstream.

Assessors should also be aware that within a single impact source (e.g. a scientific publication) impacts of alien species can be reported at different community scales and should be scored as such within an assessment. For example, in Mujingni (2012) the effects of the alien water hyacinth (*Eichhornia crassipes*) were assessed for 16 villages within five regional areas in Cameroon. As the author conducted the surveys at each village and

communicated the results for each village individually, SEICAT results can and should ideally be reported for each village individually. These can be entered as separate observations (rows) in an assessment sheet, facilitating data usability for end-users. However, impact data will not always be reported at the level of individual communities and information is often summarised across surveyed communities (i.e. regions). In these cases, increasing uncertainty as to whether the impacts to the community of interest are appropriately captured in the impact score can be reflected by assigning lower confidence.

It is important to mention that impacts should not be linked to specific localities. Rather, impacts should be linked to the people that comprise the community of interest since it is them performing the activities that are the focus. For instance, if an alien species renders a specific area unsuitable for an activity to be performed, people may be able to compensate for this by performing the activity elsewhere. Take a hypothetical example where an alien algal species invades a local lake (Lake Sykat) – which is a popular location for freshwater scuba diving – causing a significant reduction in water clarity. At this lake, the activity of freshwater scuba diving is completely abandoned as a result of the alien species. Incorrectly linking the activity to a location could then lead an assessor to the incorrect impact score of Major. However, within the local area, there are several other lakes where the alien species is absent, meaning not all people actually abandon the activity. Thus, there may be two potential scenarios here. In scenario one, all people who previously used to dive at Lake Sykat (i.e. our community of interest) now continue to dive at the other lakes. In this case then, the appropriate impact score would be Minor because the activity is still carried out but not in the preferred location so there is an additional degree of difficulty in performing the activity (e.g. it may take longer to get to the alternative lakes and is therefore associated with an additional cost in fuel and time, or other lakes are not as diverse or beautiful for diving). In scenario two, some people who previously used to dive at Lake Sykat continue to do so but some decide to stop diving altogether, in which case the impact score would be a Moderate.

The temporal scale of an impact measurement

Temporal variability represents a major challenge in obtaining representative measurements that accurately describe the impacts of alien species (Sapsford et al. 2020). If a measurement is taken over a time period that does not capture the true impact magnitude of an alien species, impacts can be either over- or under-estimated. Temporal effects impacting humans in terms of both beings and doings most often persist because of the life-history and phenological traits of alien species that are associated with time. As such, impacts are not necessarily constant. For example, this is seen when human health is impacted at a specific time of year because of pollen allergies caused by alien weeds (Bernard-Verdier et al. 2022) or due to increased abundances of species that can be of medical concern, such as insects with venomous or urticant properties like vespid wasp species or oak processionary moth (McGain et al. 2000; Battisti el al. 2017). Thus, maximum impacts will not be well captured if measuring the effects of alien species to human well-being does not coincide with the relevant time during which the impact occurs, and this should be captured in the confidence score.

To quantify impacts related to doings (see earlier section), information on activities performed by the affected communities needs to be available in order for assessors to evaluate how these activities have changed. Ideally, this would include baseline knowledge on how frequently the activity was conducted by individuals prior to the arrival of the alien species (Fig. 5). Very few activities are carried out continuously and activity patterns differ among individuals and activities. Some may be conducted regularly, for instance, on a daily basis, such as walking to work, whereas other activities may occur more ephemerally or erratically over larger time periods (e.g. monthly or annually) and not necessarily at regular intervals. Likewise, certain activities will only take place during specific seasons (e.g. recreational activities like skiing, agricultural activities). In order to determine if some individuals have abandoned a particular activity (i.e. a Moderate impact) or whether an entire community of interest has abandoned the activity (i.e. a Major or Massive impact) some knowledge about activity patterns is required.

Activity patterns can differ in terms of the frequency, duration and periodicity (Fig. 6). The frequency and duration relate to how often an activity is carried out, and for how long, respectively, whereas the periodicity refers to the trends or reoccurring
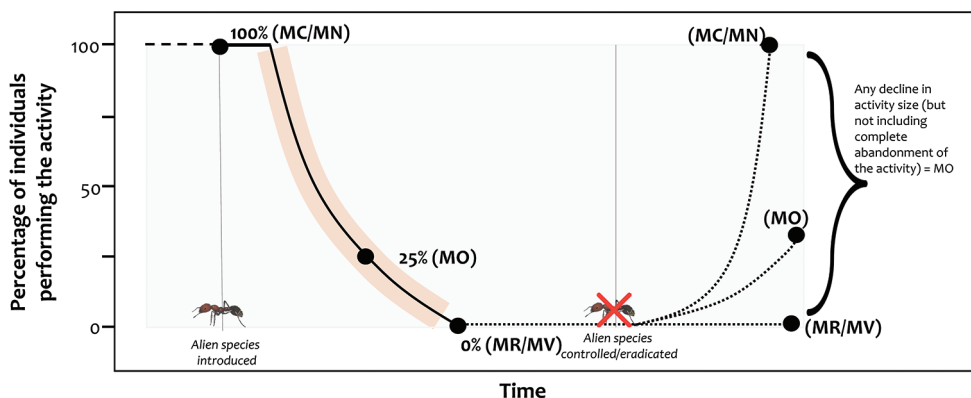

Figure 5. Impact magnitude can change over time. Conceptual drawing illustrates how the impact category depends on the percentage of people in the community of interest that continue to perform an activity in relation to the arrival, and subsequent management, of an alien across time. To accurately measure impact in terms of the effects on human activities, we must know the number (and ideally, the individual identity, see also Fig. 4) of people in our community of interest that are performing the activity prior to the alien being introduced. When all people are still performing the activity (i.e. 100% of people that would perform the activity in the absence of the alien still perform the activity in the presence of the alien), the impact score is restricted to either Minimal Concern (MC) or Minor (MN). However, any decline observed in the activity size – that is the number of people performing an activity – is assigned a Moderate (MO) (demonstrated by brackets and shaded orange area of line) until the point at which no people continue with the activity (Major/Massive; MR/MV). Impacts are subject to temporal variation due to life-history and phenological traits of alien species and dynamics of human activities. Taking snapshots at certain points (represented by large black dots) will lead to certain impact scores based on SEICAT criteria. In this figure, how the impact score could theoretically change over time if management of the alien species population commences is demonstrated with the dotted lines. Symbol obtained from the Integration and Application Network (ian.umces.edu/media-library).

variation in when an activity is performed and is often thought of as seasons or cycles. Understanding periodicity is therefore important to understand if an activity has been abandoned. However, measuring impacts of alien species is often restricted to sampling within short, discrete temporal periods, often referred to as 'snapshots' (Crystal-Ornelas and Lockwood 2020), due to logistic restrictions limiting the feasibility of longer-term data collection. Understanding whether such snapshots accurately characterise the true impact of an alien species will depend on the time interval and the timing of the onset of measurements. If the timescales used to measure potential changes in activity are too short, it may give the assessor the impression that activities have been abandoned by some people when in actuality people are just performing them less frequently (which would be a Minor impact category) (Fig. 5). Take, for example, a situation where an alien jellyfish that blooms over a period of a few weeks leads to the majority of people giving up activities in the water during that time, but the activities resume after the jellyfish disappear again. This impact should be scored as Minor as the activity resumes across the recorded time scale despite the fact that there was a period of time in which all members of the community of interest completely abandoned their water activities.

Also relevant to activity patterns are where changes to the frequency and/or duration of activities occur as a result of an alien species. Whereas some individuals may perform an activity for a shorter duration each time because of the alien, others may have to spend a longer time performing the activity because of the alien as it makes an activity more arduous to obtain the same previous result. For instance, people may spend
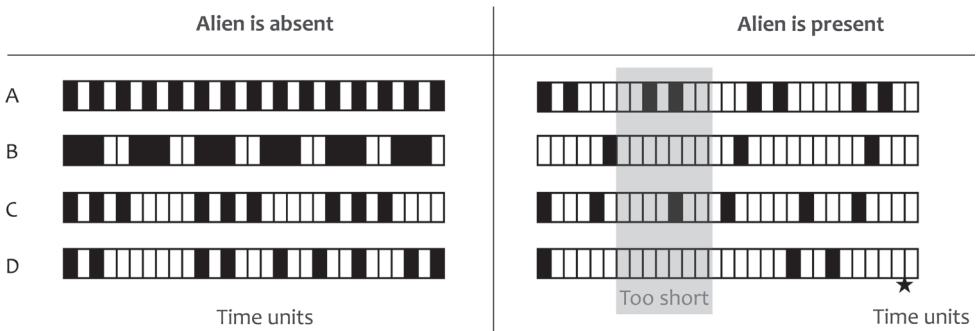
Figure 6. Examples of the frequency in which four people (**A–D**) participate in an activity across time, where black cells indicate the activity being performed at that point in time. The individual activity patterns are seen on the left panel when the alien species is absent, and on the right panel when the alien species is present. If the timescale over which the change in activity is evaluated is too short, the true impact may not be accurately identified. In this example, all people abandon the activity at the point indicated by the star (although prior to this their duration and frequency of performing the activity may have changed as a result of the alien), yet the measurement is made in the shaded area. Note that it is unlikely that people will change the activity patterns immediately in response to the arrival of an alien species as the abundance will be low and therefore impacts will not be pronounced. Here, because the timescale in which the change to activity was measured is too short, the relevancy to activity patterns of people is not realised. Based on the timescale the measurement was made, it may be assumed that individuals **A** and **C** continue the activity whilst individuals **B** and **D** have stopped it entirely.

less time participating in activities outdoors because of alien mosquito or wasp species, or anglers may have to spend more time fishing in order to catch the same number of fish they had previously, as an alien species is causing negative effects to the fish population. Impact reports detailing such changes but with no indication that individuals stop performing the activity altogether should always be reported as Minor (Table 1, Fig. 2).

Terms used to describe impact categories

The current terms used to describe impact categories are intended to reflect the increasing severity of impact that alien species have on human well-being. Yet the usage of these terms could be problematic as they may be interpreted differently by different people, therefore introducing an additional source of subjective judgement in the scoring process (see Probert et al. 2020). For example, even impacts classified as "Minimal Concern" or "Minor" may be sufficiently large to apply management, although both terms may suggest they are not significant enough to warrant action. Moreover, there is concern that the terms could become misused and misrepresented to suit political agendas in biodiversity management and decision-making. Arguments in support or opposition of species management should not be based on SEICAT scoring in isolation of the critical contexts in which impacts occur.

As such, the usage of more neutral terms for each impact category could help address the issue of terms being used improperly. One option would be to rename categories numerically, where the current descriptors of Minimal Concern (MC), Minor (MN), Moderate (MO), Major (MR) and Massive (MV) are replaced with Category 1 (C1), Category 2 (C2), Category 3 (C3), Category 4a (C4a) and Category 4b (C4b), respectively. The decision to assign the two highest impact scores of Major and Massive with Category 4a and Category 4b reflect the situation where both categories represent when an entire activity has been abandoned, with the only difference being that Massive is (hypothetically) irreversible, wherein even if the alien species were removed, people would not commence participating in the activity again. Whilst such categories still imply an ordinal scale of impact, the use of more neutral terminology reduces the potential of more value-laden categories being politicised in management decisions and may be less-prone to eliciting subjective judgement during the assessment process. Compared to other frameworks that adopt these categories for scoring biodiversity impacts (IUCN 2020; Vimercati et al. 2022) this is of particular importance given that activities differ vastly in their importance for human well-being and the ethical implications of misusing the qualitative terminology.

Generating detailed and transparent SEICAT assessments

A primary recommendation for future assessments is to adopt an open-data policy. This is required to promote transparency and to generate broadly accessible and useful information. At a minimum, research using the SEICAT framework should ensure

data records are available upon publication (i.e. not only the maximum score for each species) and that each scored impact is accompanied with the source reference, impact and confidence scores, and quotation(s) supporting the assessment. However, there is additional information that may be available for each impact report that if included, would generate even more comprehensive and useful impact assessments.

The new additional information recommended as columns in the SEICAT spreadsheet include: type of impact report (e.g. survey, observation), spatial scale (e.g. national, regional), and clearly separates the impacts to preferred activities and those impacts that lead to compensatory or burdensome activities (see Suppl. material 1). Assessors are also encouraged to detail in a notes section any additional information that is valuable when available, such as whether impact information is inferred, or whether the impact is subject to seasonal variation. For observations that are not relevant to SEICAT (e.g. a report of a positive impact), an additional column, non-scorable justification, is included. These additional variables are intended to make SEICAT assessment data more useful and user-friendly.

To demonstrate the proposed refinements and recommendations, we use SEICAT assessments (see Suppl. material 1: worksheet C) for a range of different alien taxa, selecting examples that affect different constituents of human well-being at different global localities. Using these data, we provide an exemplary data collection template for future SEICAT assessors to use. The increased level of detail that we suggest assessors record when evaluating the impact of an alien species is intended to provide more context for end-users of SEICAT assessments and remove the necessity to re-examine impact records to make more meaningful intra- and inter-specific comparisons. It is important to understand that our suggestions for specific improvements should not be perceived as the endpoint for assessments. Future improvements to the SEICAT framework and its application are likely necessary to further advance our ability to capture and compare socio-economic impacts under different invasion scenarios.

Future directions: structuring surveys to capture SEICAT data

Data availability is one of the limiting factors to applying SEICAT across different taxonomic groups. For instance, in a global analysis of alien bird species, only 14% of birds assessed yielded impact reports (Evans et al. 2020). Similarly, for one of the best studied groups of alien trees, Australian acacias, only 19 impact records were found for SEICAT (Kumschick and Jansen 2023). Impacts of most alien species are generally not well understood, although there is a growing body of literature for some environmental and economic contexts (Gallardo et al. 2015; David et al. 2017; Diagne et al. 2021; Dueñas et al. 2021). One general exception may be alien taxa of medical concern, such as species with toxic or venomous properties or vectors of disease, whose impacts tend to be documented in the medical literature (e.g. see Galanidi et al. 2018). Other recorded impacts to various facets of human well-being can be found (Shackleton et al. 2019), although environmental impacts in general tend to be more frequently reported (Measey et al. 2020; Allmert et al. 2022). Furthermore, language barriers reduce the accessibility of impact data for alien species (Angulo et al. 2021) and thus

future SEICAT assessments can benefit greatly from targeted literature searches in the local language where alien species are likely to be affecting communities. For instance, in their assessment of invasive fishes in the Mediterranean, Galanidi et al. (2018) obtained 17% of their impact scores from non-English sources. To overcome some of the challenges associated with data availability, we posit that a key area for future research lies in generating new socio-economic impact data.

Unlike ecological impact studies, which generally require field observation and experiments to effectively quantify the effects of alien species, understanding the socio-economic impacts of alien species can be facilitated through questionnaires and interviews with people. These tools allow researchers to directly ask (potentially) affected people about their experiences and perceptions. Questionnaires can be developed with SEICAT criteria in mind meaning that true impacts based on SEICAT's semi-quantitative scale can be effectively captured with relatively low uncertainty if robust survey methods are adopted. Surveys may allow the rapid-generation of data for alien species that may help expedite decision-making processes, which is especially crucial given another major source of uncertainty stems from temporal biases in alien species impact reporting, where there are distinct lags between the alien species establishment, impacts, and impact reporting (Pyšek et al. 2008; Hulme et al. 2013). Future research should be invested into what are the most suitable methods and study designs to capture different social contexts and impact types.

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Supplementary material 1

SEICAT assessment supplementary material
Authors: Anna F. Probert, Giovanni Vimercati, Sabrina Kumschick, Lara Volery, Sven Bacher
Data type: xlsx
Explanation note: Template reporting for SEICAT assessments.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/neobiota.89.109911.suppl1

RESEARCH ARTICLE

Headwater refuges: Flow protects *Austropotamobius* crayfish from *Faxonius limosus* invasion

Alina Satmari[1*], Kristian Miok[2*], Mihaela C. Ion[3], Claudia Zaharia[4], Anne Schrimpf[5], Lucian Pârvulescu[2,6]

1 *Department of Geography, Faculty of Chemistry, Biology, Geography, West University of Timisoara, Vasile Pârvan 4 Bd. 300223 Timisoara, Romania* **2** *Advanced Environmental Research Institute, West University of Timisoara, Vasile Pârvan 4 Bd. 300223 Timisoara, Romania* **3** *Institute of Biology Bucharest, Romanian Academy, 060031 Bucharest, Romania* **4** *Department of Mathematics, Faculty of Mathematics and Computer Science, West University of Timisoara, 300223, Timisoara, Romania* **5** *University Kaiserslautern-Landau, Institute for Environmental Sciences, 76829 Landau, Germany* **6** *Department of Biology-Chemistry, Faculty of Chemistry, Biology, Geography, West University of Timisoara, 300115 Timisoara, Romania*

Corresponding author: Lucian Pârvulescu (lucian.parvulescu@e-uvt.ro)

Abstract

This study explores the geospatial relationship between the invasive crayfish species *Faxonius limosus* and the native *Austropotamobius bihariensis* and *A. torrentium* crayfish populations in Eastern Europe, identifying the environmental factors which influence the invasion. We used species distribution modelling based on several climatic, geophysical and water quality variables and crayfish distributional data to predict sectors suitable for each species within the river network. Thus, we identified the sectors potentially connecting invasive and native population clusters and quantified the degree of proximity between competing species. These sectors were then extensively surveyed with trapping and hand searching, doubled by eDNA methods, in order to assess whether any crayfish or the crayfish plague pathogen *Aphanomyces astaci* are present. The predictive models exhibited excellent performance and successfully distinguished between the analysed crayfish species. The expansion of *F. limosus* in streams was found to be limited by flash-flood potential, resulting in a range that is constrained to lowland rivers. Field surveys found neither crayfish nor pathogen presence in the connective sectors. Another interesting finding derived from the screening efforts, which are among the most extensive carried out across native, apparently healthy crayfish populations, was the existence of a latent infection with an *A. astaci* strain identified as A-haplogroup. Our results provide realistic insights for the long-term conservation of native *Austropotamobius* species, which appear to be naturally

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*   These authors contributed equally to this work.

protected from *F. limosus* expansion. Conservation efforts can thus focus on other relevant aspects, such as ark-sites establishment for preventing the spread of more dangerous invasive crayfish species and of virulent crayfish plague pathogen strains, even in locations without direct contact between crayfish hosts.

Introduction

Invasions of non-native species are a global phenomenon affecting almost every region and taxonomic group (Catford et al. 2012; Essl et al. 2020). Although the knowledge of invasions continues to improve, efforts to stop or slow them have had limited success (Clavero and García-Berthou 2005; Crooks 2005; Wilson et al. 2009). To preserve remaining native populations, a practical and low cost approach is to find "ark-sites", areas where invasions are unlikely to reach in the existing conditions or with minor interventions (Holdich et al. 2009; Nightingale et al. 2017). Our goal in this study is to assess the vulnerability of the native *Austropotamobius bihariensis* Pârvulescu, 2019 and *A. torrentium* (von Paula Schrank, 1803) crayfish populations in the natural conditions offered by the mountain habitats in Romania, in light of the ongoing invasion of *Faxonius limosus* (Rafinesque, 1817).

The impact of crayfish invasions on native species populations can manifest as declines in density (Gherardi et al. 2011), changes in community structure (Kuhlmann 2016; Galib et al. 2021), or in trophic position (Pacioglu et al. 2020; Chucholl and Chucholl 2021), and even habitat alteration (Gherardi 2007; Albertson and Daniels 2016). Invasive crayfish can exhibit plasticity and can employ various tactics to increase their chances of success. Some of these are: making use of superior phenotypic traits (Chuang and Peterson 2016; Messager and Olden 2019), changing reproductive strategies (Pârvulescu et al. 2015; Francesconi et al. 2021a), acting as pathogen reservoirs and transmitting disease (Strauss et al. 2012), or even using sheer boldness (Hudina et al. 2015; Pârvulescu et al. 2021; Roessink et al. 2022).

While significant international efforts have gone towards regulating probable invasion entry points through legislation, the prevention and control methods available in natural habitats are still quite limited. Measures such as building mechanical barriers (e.g., dams) to block upstream movement can be effective in some specific conditions, but these are not stopping the invasions completely (Krieg and Zenker 2020; Krieg et al. 2020, 2021). Commercial activities such as food and pet trade or leisure activities (Andriantsoa et al. 2020; Bláha et al. 2022) and even animal-mediated spread of non-native taxa (Águas et al. 2014; Anastácio et al. 2014) can facilitate the invasion of some crayfish species. On top of that, the presence of the *Aphanomyces astaci* pathogen carried by most of the invasive crayfish species (Mrugała et al. 2015) makes prevention an urgent matter. However, it should be pointed out that some native crayfish

populations have shown signs of long-term survival and coexistence with invasives. For example, the *Pontastacus leptodactylus* Eschscholtz, 1823 seems to have good resistance to the crayfish plague pathogen (Panteleit et al. 2018; Ungureanu et al. 2020) and has reportedly lived alongside invasive crayfish for a long time, showing signs of recovery (Pacioglu et al. 2020). Despite analyses based on large datasets, a clear temporal trend has not been established for crayfish invasions in Europe (Soto et al. 2023).

Faxonius limosus originates from North America and was introduced to Europe for commercial reasons in the late 19th Century (Holdich 2002). It has since spread widely in Western Europe, reaching Romania in 2008 (Parvulescu et al. 2009). This species is harmful to the native crayfish populations (Gherardi et al. 2011; Capinha et al. 2013; Aluma et al. 2023), as it competes with them for food and habitat (Lele and Pârvulescu 2017; Veselý et al. 2021), and also brings the fatal crayfish plague disease pathogen (Pârvulescu et al. 2012), which is listed among the 100 worst alien species in the world (Luque et al. 2014). The pathogen is almost permanently present alongside invading populations or even far ahead of the invaded areas (Schrimpf et al. 2012; Panteleit et al. 2018; Ungureanu et al. 2020).

Our focus is to find if there is a favourable spatial and ecological context for the invasion of *F. limosus* to progress into the habitats of the two *Austropotamobius* native species naturally living in the upper regions of mountain ranges in Romania, and thus, to assess how protected the native populations are from this invasion. To this aim, we used several climatic, geophysical and water quality variables known to be relevant in describing crayfish distribution, as well as species presence/absence data, to predict the most suitable river sectors occupied by the targeted species, and checked for proximity and potential spatial overlap between the invasive and natives.

Methods

The study was designed in two stages. The first stage involved species distribution modelling (SDM) aimed to learn ecological preferences of the assessed species. This allowed us to predict which sectors would be suitable for each species within the river network determined by the areal of *A. bihariensis* and *A. torrentium* in Romania. The second stage identified the sectors potentially connecting invasive and native population clusters and quantified the degree of proximity between competing species to identify areas of concern. These areas were then visited in the field, using trapping and hand searching, doubled by modern molecular techniques, to detect crayfish and the crayfish plague pathogen, *A. astaci*.

Crayfish distribution data

Special attention was given to the location data of crayfish presence and absence (Fig. 1A). Although citizen science is increasingly popular, its reliability is still debated (Zizka et al. 2020), which led us to rely solely on scientific publications. Our dataset
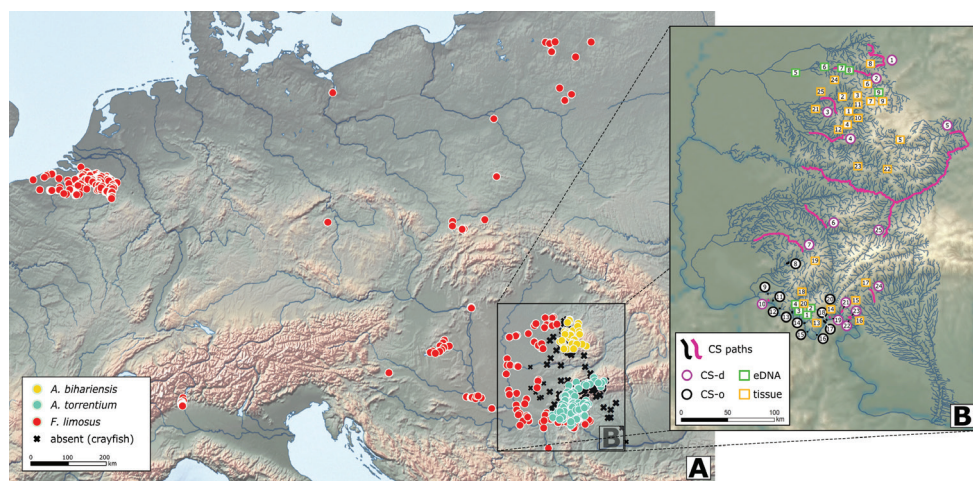
Figure 1. A sites with crayfish presence/absence data used for training SDM **B** paths connecting closest river sectors with positive predictions for native and invasive species (codes according to Table 4), and crayfish plague tissue and eDNA sampling locations (codes according to Suppl. material 3 for eDNA and Table 5 for tissue samples).

comprised existing data from previous studies on native *Austropotamobius* crayfish in Romania (Pârvulescu and Zaharia 2013; Pârvulescu 2019; Pârvulescu et al. 2020) and literature data from the first detection of *F. limosus* invasion in this region (Pârvulescu et al. 2009) to the latest publication (Pacioglu et al. 2020). As Romania is still in the early stage of the invasion of *F. limosus*, we expanded the dataset with available data in Europe from published literature. Additional new data was collected in the field during 2019–2022 to get the most up to date situation in the investigated area. We captured crayfish by using hand searching for native species, and baited traps (Pirate type, with double entrance) for the invasive species. Capture effort was set as one daylight visit per site for hand searching, visiting a river sector of at least 150 m, whereas trapping was applied for at least two nights with batches of 3 traps per site. Data on crayfish absences were obtained exclusively from field investigations (Fig. 1).

Geospatial database

Selecting the most important variables that will be included in the model and on which certain decisions will be made is essential and not always trivial (Mac Nally 2002; van de Pol et al. 2016; Fourcade et al. 2018). We considered variables whose relevance in predicting crayfish distributions has already been demonstrated in the literature (Chucholl and Schrimpf 2016; Krause et al. 2019; Pârvulescu et al. 2020; Dornik et al. 2021; Soto et al. 2023), focusing on elevation, habitat quality, substrate, and climatic variables.

Data was processed using ArcGIS Pro software (ESRI, Redlands) and Saga 8.5.0. (https://saga-gis.sourceforge.io/en/index.html). Elevation data, at 3 arc-second

(90 m × 90 m) spatial resolution digital elevation model (DEM), was downloaded from USGS Earth Explorer data portal (https://earthexplorer.usgs.gov/). The DEMs were first combined into a raster mosaic for the whole area of interest. ArcGIS Pro Hydrology Tools were used to create the stream network by modelling the flow of water across the raster surface.

Regarding environmental factors used as predictors, we used a set of 12 variables that described the ecological, climatic and edaphic conditions. Altitude (ALT) was sampled at each point location from the DEM. Annual mean temperature (BIO1) and another six climatic variables, considered relevant for the species in question, were downloaded from the WorldClim data portal (https://www.worldclim.org/). This database provides free historical climate data for 1970–2000 (Fick and Hijmans 2017). The used variables were the maximum temperature of the warmest month (BIO5), the mean temperature of the driest quarter (BIO9), the annual precipitation (BIO12), the precipitation of the wettest quarter (BIO16), the precipitation of the driest quarter (BIO17) and the precipitation of the warmest quarter (BIO18). Soil type (TYS) was extracted from a raster layer with data on the soil classes based on the international standard for soil classification system World Reference Base (Hengl et al. 2017). We obtained this raster from the SoilGrids portal (Poggio et al. 2021). The thickness of soil up to the bedrock (THS) was extracted from the world-level estimated model provided by (Pelletier et al. 2016). The 30 arc-second climatic and soil data were sampled for each point location of our study dataset.

Remote Water Quality (RWQ) is an ecological index that measures the anthropogenic pollution potential in the upstream areas (Şandric et al. 2019). For its computation, the 44 third-level inventory classes of CORINE Land Cover 2018, (https://land.copernicus.eu/pan-european/corine-land-cover/clc2018) were regrouped into 4 new classes based on their human-based potential impact (Burkhard et al. 2012). RWQ represents the average of class scores weighted by their specific watershed area. We ran an iterated chain of specific steps using Model Builder. This process delineates unique watersheds and computes the corresponding RWQ index at each point location at 3 arc-second (90 m × 90 m) resolution.

Flash-flood potential (FFP) estimates stream disturbance potential according to the local and upstream drainage velocity (Pârvulescu et al. 2016). This index is calculated starting with the river network and two land-surface variables: slope gradient (accounting for the potential water velocity at a given site) and catchment slope (estimating the average gradient of a surface that drains towards a given site, thus accounting for the potential of flash floods as a consequence of heavy rain events). Higher values of FFP indicate a high risk of water velocity increasing rapidly and temporarily. The slope was derived in ArcGIS Pro using the Spatial Analyst Slope tool, in a 3 × 3 window neighbourhood, using the 3 arc-second (90 m × 90 m) previously processed DEM mosaic. The spatial resolution of output data was preserved at 90 m × 90 m. The catchment slope was calculated using Saga hydrological tools. FFP was computed in each river network cell location as the product of slope gradient and catchment slope, using ArcGIS Pro raster mathematical tools.

Species distribution modeling and prediction

In order to understand the interaction between the two *Austropotamobius* species and *F. limosus*, both the locations for presence, as well as absence of crayfish, are essential. The data for each of the three species were analysed separately using machine learning modeling techniques. The dataset for *A. bihariensis* contained 106 records (31 presences and 75 absences), the one for *A. torrentium* had 213 records (93 presences and 120 absences), while the one for *F. limosus* had the largest sample size with 711 records (482 presences and 229 absences). The species presence was predicted using Random Forest (RF) method on the 12 input (predictor) variables, using the "sklearn" package in Python. To obtain the best fitting random forest architecture we performed hyperparameter tuning for each dataset.

The prediction task was done in several stages. The first step was to reveal which of the predictor variables have relevance on predicting the crayfish locations, i.e. feature importance (FI), based on the mean decrease in impurity. In the second step we trained machine learning models using the most important variables detected above. For each of the three crayfish species we used Scikit-Learn's GridSearchCV that evaluated various hyperparameter candidates from the grid of parameter values. The hyperparameters considered were the number of trees to be used in the model, the maximum features in each tree, the maximum number of splits each tree can take and how many divisions of nodes should be done. The best solutions are presented in Suppl. material 1. Once the models were fitted, they were used to predict crayfish presence/absence on the full river network for each of the three species. In each case, the prediction cut-off was set to a default value of 0.5. Predicted suitable ranges (SR) were calculated using partial dependence plots to understand the variable intervals on which each species fit the best. Observing high compliance between SRs and observed ranges (OR) provides us with noteworthy information that SRs can be trusted and used for further interpretation (Cutler et al. 2007). Additionally, to understand the species' relationship with each variable retained in the model, we calculated the percentage of overlap of the SR from the total range (TR) of the variable (further denoted %Ov). The distributions of relevant ecological variables were compared between the investigated crayfish species using two samples Welch's t-test.

Finally, the hydrographic network dataset used in the study was loaded into GIS software to identify positive predictions for each native and invasive species with the aim to observe and understand each species spatiality. We compared the positively predicted river network between the two *Austropotamobius* species, and between each and the invasive *F. limosus* separately, determining the degree of overlap.

Concerning situations for contact zones between native and invasive crayfish species

To identify potential areas of concern for the colonization of native *Austropotamobius* crayfish populations by the invasive species *F. limosus*, we identified continuous network sectors with positive prediction for one of the native species that contained at least one confirmed presence point for that species. Subsequently, following the river

course, each such sector was associated with the closest river sector with positive prediction for the invasive species, thus defining "paths".

Based on these paths, we measured the level of separation (LoS). It serves as a proxy for the "ecological cost" representing the sum of challenges that migrating individuals would encounter at each point within the habitat (Zeller et al. 2012). The LoS was determined by summing the differences between the threshold (0.5) and the predicted probability of *F. limosus* occurrence for each network cell along the path. We then computed the LoS per kilometer of river path for better comparability. Two cases are possible: (i) LoS > 0 (implying that a physical distance existed along the path between two sectors), and (ii) LoS ≤ 0 (when the two sectors overlapped).

Situations belonging to the first category were further analysed by field investigations aimed at detecting whether any crayfish or *A. astaci* were present in the area. In-field crayfish searches used manual methods or traps as described above. In some instances, we also collected water filtrate (as in Shogren et al. 2017) by filtering three replicates (minimum of 5 L of water each) per site, one replicate from the center of the river channel and two close to the riverbanks. These water samples were taken to give a better resolution assessment through eDNA analysis (Seymour et al. 2021). In total, 27 filters from 9 sites, including Oradea as control (see Fig. 1B), were collected in the summer season to enhance detectability success (Baudry et al. 2023), and stored in ethanol. A high salt DNA extraction method (modified after Aljanabi and Martinez 1997) was applied for DNA isolation from the filters. For the qPCR analysis for each sample 6 µl Environmental PCR Master Mix, 1.6 µl nuclease-free water and 0.4 µl of each forward and reverse primer and the probe were mixed. Then in total 11 µl of this mixture were added to a PCR tube. In each tube, 1.2 µl of DNA from each sample was added. For the detection of *F. limosus* DNA the primers and probe from Rusch et al. (2020) were used, for the stone crayfish DNA the primers and probe from Chucholl et al. (2021) were used and for *A. astaci* DNA the above-mentioned primers and probe were applied.

Screening of native crayfish populations

Since none of the in-field investigations of paths with LoS > 0 found any crayfish, we extended the search upstream, in known native population sites. The goal was to verify whether *A. astaci* had spread there, even without *F. limosus*. Native crayfish were captured and sampled (see Fig. 1B) by collecting a piece of the uropod, to harm the crayfish as little as possible. Exuviae or dead animals were collected as well where available. Samples were stored in 96% ethanol. In total, 353 samples from 25 crayfish populations (12 of *A. bihariensis*, 6 of *A. torrentium* and 6 of *Astacus astacus*, and one mixed population of *A. torrentium* and *A. astacus*) were used for the crayfish plague analysis. In the laboratory we applied a CTAB DNA extraction protocol modified after Vrålstad et al. (2009). The whole uropod was used; additionally, in the case of exuviae or dead animals, other parts of the carapax were used as well (Vrålstad et al. 2009). For the real-time PCR a new set of primers and probe was used according to Francesconi et al. (2021b) and the annealing times and temperatures were also adapted.

In order to identify the *A. astaci* haplotype, samples with a high ct-value were selected to be sequenced using two primer pairs amplifying the mitochondrial ribosomal rnnS (AphSSUF and AphSSUR) and rnnL (AphLSUF and AphLSUR) according to Makkonen et al. (2018).

Availability of data

The data underpinning the analyses reported in this paper are deposited in the Elsevier's Mendeley Data repository at https://doi.org/10.17632/5vg35hc58m.2.

Results

Species distributions modeling and prediction

The SDMs performed well in predicting the occurence of native species (Fig. 2), even with the small sample size in the particular case of the endemic *A. bihariensis*. The relevance of predictors for each species can be found in Tables 1–3 providing a visualisation of the predictor influence for each species. The important predictors for *A. bihariensis* included ALT, FFP, RWQ and several climatic variables (BIO1, BIO5, BIO16, BIO12, BIO9) providing an accuracy of 85.2% (F1 = 0.745). For *A. torrentium*, the important predictors were RWQ, BIO17, ALT, BIO12, FFP, BIO1, BIO5, BIO16 and BIO9, with the RF classifier providing an accuracy of 85.4% (F1 = 0.851). In the case of *F. limosus*, the method performed the best, with a high accuracy 99.5% (F1 = 0.99) using the predictors FFP, ALT, BIO9, BIO16, THS, and BIO1. Notably, variables related to soil had a low influence on the accurate location detection for both *Austropotamobius* species, whereas water quality (RWQ) was found to be one of the most important predictors for both species. For the invasive species *F. limosus*, the variables RWQ, BIO5, BIO12, and BIO17 had very limited influence on prediction.

We found an overlap of 27.2% of the predicted river network of *A. torrentium* over *A. bihariensis* (see Fig. 2A), and 17.5% of *A. bihariensis* over *A. torrentium* (see Fig. 2B). Furthermore, the overlap of the prediction of invasive species over either of the two native species was minimal, with 0% of the predicted network for *A. bihariensis* and 0.88% for *A. torrentium*.

Although the SR and OR were highly consistent, the species %Ov (Tables 1–3) revealed how each species could explore the ecological niche within a geographic area as indicated by the TR for each variable. *Austropotamobius* species only tolerated the lower values of the RWQ, with 20.44 %Ov (0.3–1.2) for *A. bihariensis* and 27.26 %Ov for *A. torrentium* (0–1.2) out of the TR found in the study area. Also notably, there were differences in altitude between the natives and invasive species, with *A. bihariensis* exploring only 28.31 %Ov (between 259 to 796 m asl) of the range in the region, while *A. torrentium* was found slightly more permissive in its lower altitude margin, with an SR ranging from 120 to 798 m asl (35.77 %Ov), whereas *F. limosus* ranged between 0–370 m asl with 19.52 %Ov. Another difference between the species was their toler-
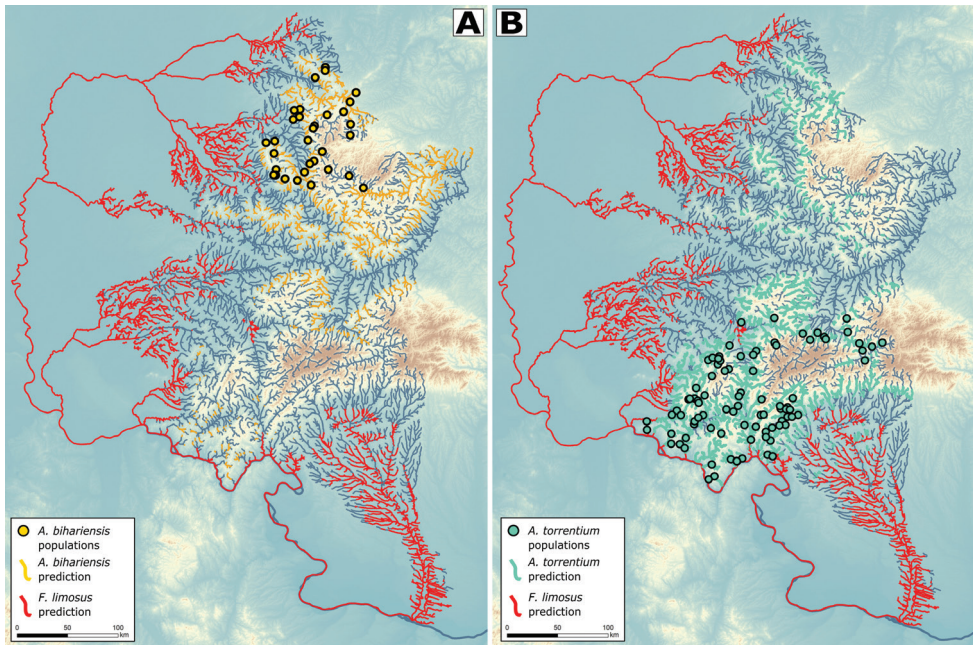
Figure 2. Prediction results over the investigated network for **A** *A. bihariensis* vs *F. limosus* and **B** *A. torrentium* vs *F. limosus*.

Table 1. Analysis of variables retained in *A. bihariensis* species distribution model: FI – feature importance; OR – observed range; SR – suitable range; TR – total range; %Ov – percentage of overlap of the SR from the TR. For more information on habitat variable codes, we refer readers to the "Geospatial database" section in Methods.

Variable	FI	OR	SR	TR	%Ov
BIO1	0.164	5.09–9.5	5–9.8	0.2–12.3	39.68%
ALT	0.120	259.4–935.6	259.4–796	0–1921	28.31%
BIO5	0.114	20.5–25.5	19.3–25.6	13.8–30.2	38.41%
BIO16	0.105	229–311	230–280	166–386	22.72%
BIO12	0.096	637–826	520–730	491–1047	37.76%
BIO9	0.094	-2.8–1.4	-2.8–1.5	-7.5–17.7	16.98%
FFP	0.089	0.18–1.7	0.2–1.8	0–12.27	13.03%
RWQ	0.086	0–1.01	0.3–1.2	0–4.40	20.44%

ance for temperature, as *A. torrentium* had a much lower %Ov for variables describing temperature (BIO1, BIO5, BIO9) in comparison with *A. bihariensis*. Important differences were observed between the natives and invasive species regarding temperature variables (BIO1, BIO9), which were considerably higher for the invasive species (see Tables 1–3). Similar differences were observed for climatic variables describing precipitation BIO12 with much higher requirements for *A. bihariensis* and BIO17 for *A. torrentium*. BIO16 was found relevant for both native species and had a high %Ov. A noticeable difference between native *Austropotamobius* and invasive *F. limosus* was

Table 2. Analysis of variables retained in *A. torrentium* species distribution model: FI – feature importance; OR – observed range; SR – suitable range; TR – total range; %Ov – percentage of overlap of the SR from the TR. For more information on habitat variable codes, we refer readers to the "Geospatial database" section in Methods.

Variable	FI	OR	SR	TR	%Ov
RWQ	0.138	0–1.03	0–1.2	0–4.40	27.26%
BIO17	0.132	106–142	114–145	73–175	30.39%
ALT	0.126	119.4–868.7	120–798	0–1921	35.77%
BIO12	0.122	642–828	650–775	491–1047	22.48%
FFP	0.104	0–4.3	0.2–3	0–12.27	22.81%
BIO1	0.072	6.2–11.2	6.9–10.8	0.2–12.3	32.24%
BIO5	0.065	20.9–28.3	22.3–27.8	13.8–30.2	33.53%
BIO16	0.063	207–295	170–296	166–386	57.27%
BIO9	0.062	-1.7–2.7	-1.1–2.5	-7.5–17.7	14.21%

Table 3. Analysis of variables retained in *F. limosus* species distribution model: FI – feature importance; OR – observed range; SR – suitable range; TR – total range; %Ov – percentage of overlap of the SR from the TR. For more information on habitat variable codes, we refer readers to the "Geospatial database" section in Methods.

Variable	FI	OR	SR	TR	%Ov
FFP	0.372	0–1.229	0–0.94	0–12.27	7.66%
ALT	0.216	0–358	0–370	0–1921	19.52%
BIO9	0.096	-2.9–6.6	-0.8–6.6	-7.5–17.7	99.53%
BIO16	0.088	168–383	168–383	166–386	97.72%
THS	0.081	1–50	0–50	0–50	100%
BIO1	0.067	5.8–12.2	6.5–12.3	0.2–12.3	47.95%

particularly evident for FFP, with the invasive species exploring significantly less of the TR with %Ov of 7.66% (0–0.94), than *A. bihariensis* (0.2–1.8, 13.03 %Ov) and *A. torrentium* (0.2–3, 22.81 %Ov). The differences between the three species regarding the environmental parameters can also be seen by analysing the comparisons between their values in sites where each respective species was observed (Suppl. material 2).

Concerning situations for contact zones of native and invasive crayfish species

We have identified 25 paths connecting native and invasive species (Fig. 1B). Out of these, 11 were cases of LoS ≤ 0 (overlap), which are generally short, with lengths ranging from 90 to 2250 m (Table 4), being found only for the *A. torrentium* populations. As for LoS > 0 (separation by distance), we found 14 cases, with lengths ranging from 26820 to 269730 m (average 73902 m) for *A. bihariensis*, and 720 to 160560 m (average 30120 m) for *A. torrentium*. The extremes are four paths with LoS greater than 100, and five paths with a highly concerning LoS below 10 (Table 4). A better view on the adequacy of native crayfish habitats to act as ark-sites is given by LoS/Km (see Table 4), showing the difficulties faced by *F. limosus* to traverse the river sectors separating it from the natives.

Table 4. The paths and the level of separation (LoS) between sectors with positive predictions for native crayfish (*A. bihariensis* or *A. torrentium*) and the invasive *F. limosus*. LoS ≤ 0 represents overlap.

ID	Native species	Length (m)	LoS	LoS/Km
1	*A. bihariensis*	45990	135.22	2.94
2		39060	97.99	2.51
3		26820	42.72	1.59
4		41130	54.17	1.32
5		269730	716.18	3
6	*A. torrentium*	32400	71.21	2.19
7		62190	125.44	2.01
8		2160	≤ 0	-
9		180	≤ 0	-
10		990	0.65	0.66
11		540	≤ 0	-
12		540	≤ 0	-
13		990	≤ 0	-
14		810	≤ 0	-
15		90	≤ 0	-
16		180	≤ 0	-
17		2250	≤ 0	-
18		360	≤ 0	-
19		990	1.08	1.10
20		450	≤ 0	-
21		2430	3.42	1.41
22		2070	4.15	2.01
23		720	0.69	0.96
24		13950	35.89	2.57
25		160560	418.46	2.61

Screening of native crayfish populations

From a total of 27 water samples (Suppl. material 3), three were positive for *F. limosus* DNA. All three filters with water filtered from Oradea showed a positive signal in the qPCR analysis, confirming the field observations. We could not detect DNA from the two *Austropotamobius* species nor from *A. astaci* in any other filter.

The analysis of crayfish tissue samples revealed an infection rate of 64% (16 infected out of 25 investigated populations) in apparently healthy native crayfish. In total, 34 samples out of 353 (9.6%) analysed tissue samples were positive for an *A. astaci* infection (Table 5). Concerning the different species, 20 out of 191 analysed tissue samples from *A. bihariensis* (10.4%), 8 out of 83 samples from *A. torrentium* (9.6%) and 6 out of 79 samples from *A. astacus* (7.6%) were positive. Most ct-values were relatively low (35.32 to 45.96). Three samples returned results from sequencing attempts (see Table 5), one from *A. bihariensis* (site Rănușa), one from *A. torrentium* (site Plopa), and one from *A. astacus* (site Brebu), all those being detected with a ct-value suitable for sequence analysis and revealed identical to reference sequences As (named after *A. astacus*, the species on this genetic variant has been identified the first time) and Up (named after "Úpořský brook" where this genetic variant was detected the first time, on *A. torrentium*) of the A-haplogroup (Kozubíková et al. 2008; Grandjean et al. 2014).

Table 5. Results of the crayfish plague analysis of samples collected during the screening of native populations. Asterisk (*) indicates sites with genotyping results.

Species	ID	Site	GPS coordinates	Sample	Total	Positive	Negative	%
A. bihariensis	1	Boga	46.6107°N, 22.6610°E	uropods, exuvia	27	3	24	11.1
	2	Crăiasa	46.5443°N, 22.5964°E	uropods	11	0	11	0
	3	Racu	46.6631°N, 22.5255°E	uropods	13	3	10	23.1
	4	Tâlniciorii	46.4182°N, 22.4672°E	uropods, claw	13	1	12	7.7
	5	Valea Bistrii	46.4059°N, 23.0541°E	uropods	12	2	10	16.7
	6	Valea Anişelului	46.7883°N, 22.8872°E	uropods	8	0	8	0
	7	Preluca	46.7257°N, 22.8813°E	uropods	19	1	18	5.3
	8	Valea Mare	47.1242°N, 22.6216°E	uropods	28	1	27	3.6
	9	Valea Iadului	46.7447°N, 22.5597°E	uropods	20	0	20	0
	10	Cuţilor	46.8311°N, 22.3977°E	uropods	14	1	13	7.1
	11	Ciur Ponor	46.8188°N, 22.3800°E	uropods, legs	7	0	7	0
	12	Rănuşa*	46.4391°N, 22.2672°E	uropods, dead	19	8	11	42.1
total for *A. bihariensis*					**191**	**20**	**171**	**10.4**
A. torrentium	13	Sirinea	44.6387°N, 22.0863°E	uropods, dead	16	2	14	12.5
	14	Valea Satului	44.6294°N, 22.2461°E	uropods	15	1	14	6.7
	15	Jidoştiţa	44.7268°N, 22.5619°E	uropods, dead	16	0	16	0
	16	Coşuştea	44.9665°N, 22.6573°E	uropods	16	1	15	6.3
	17	Aninoasa	46.9557°N, 22.3457°E	legs	5	0	5	0
	18	Plopa*	45.0286°N, 21.8369°E	uropods	10	3	7	30
	19	Brebu	45.2288°N, 22.1436°E	uropods, exuvia	5	1	4	20
	20	Valea Poienii	44.6387°N, 22.0863°E	uropods	8	2	6	25
total for *A. torrentium*					**91**	**10**	**61**	**10.9**
A. astacus	21	Crişul Negru	46.6112°N, 22.4035°E	uropods	4	1	3	25
	22	Băcaia	46.0163°N, 23.1741°E	legs	16	0	16	0
	23	Ţebea	46.1461°N, 22.7022°E	legs	9	0	9	0
	24	Peştireului	46.9888°N, 22.4582°E	legs	20	0	20	0
	25	Valea Mare	46.6416°N, 22.2447°E	uropods	2	1	1	50
	19	Brebu*	45.2288°N, 22.1436°E	uropods, exuvia	20	2	18	10
total for *A. astacus*					**71**	**4**	**67**	**5.6**
		totals			**353**	**34**	**319**	**9.6**

Discussions

The ecological particularities of the species

From a habitat quality perspective, the *Austropotamobius* species are generally known to be sensitive (Füreder and Reynolds 2003; Demers et al. 2006; Trouilhéet al. 2007; Piyapong et al. 2020). This aspect is consistent with the results of this study, which showed the species' tolerance only for the lower range of the RWQ found in the study area, indicating low anthropogenic impact. Regarding altitude preferences, *Austropotamobius* crayfish inhabit mountain and submountain areas (Streissl and Hödl 2002; Pârvulescu and Zaharia 2013). Our study revealed that *A. bihariensis* has a preference for upstream areas at a higher altitude compared to *A. torrentium*, making the latter more exposed to contact with the invasive *F. limosus*. *A. bihariensis* has less tolerance for high temperatures and low precipitation than *A. torrentium*. This result is to be expected since it is known that, in general, karstic habitats (where this species is living) are ex-

posed to dryness (Bonacci 1993; Fiorillo and Guadagno 2010) but are more balanced with respect to temperature (O'Driscoll and DeWalle 2006; Cantú Medina et al. 2021).

The overall overlap between the prediction of invasive species and either of the native species was found to be marginal. This is most importantly due to the fact that for *F. limosus*, the FFP, which is the most relevant variable predicting the species presence, has a much lower suitable range than the other two species. Stream flow regulates many aspects of an aquatic ecosystem, increasing oxygen supply and impacting substrates, detritus, and benthic communities (Pacioglu et al. 2019a). A possible explanation for why *F. limosus* does not choose stream habitats could be from its trophic perspective (Vojkovská et al. 2014; Šidagyte et al. 2017; Pacioglu et al. 2019b; Mathers et al. 2020), considering that streams offer less diverse food which is also likely more difficult to find compared to larger rivers (Romanuk et al. 2006; Hette-Tronquart et al. 2016), possibly in relation with water velocity (Finlay et al. 1999; Light 2003; Kerby et al. 2005).

The other important variable influencing *F. limosus* distribution, altitude, is also related to stream flow velocity (Pârvulescu et al. 2016). Although the presence of the species has also been reported at higher altitudes (see Bonk and Bobrek 2020), moderate FFP can be patchy in this area (Pârvulescu et al. 2016), and therefore not optimal for invasion expansion. Those locations may actually be the result of human or other vector-mediated relocation. This is supported by other prediction approaches which show a marked decrease of presence probability for altitude over 500 m (Piyapong et al. 2020). As the native areal of the *F. limosus* is also largely oriented towards lowland habitats (Bloomer and Taylor 2020; Lieb et al. 2011), we conclude that the variables selected by our model are representative of the species' ecology.

The adequacy of habitats for conservation

In order to control invasive species, one must discover and understand habitat conditions that sustain or harm them. The findings of this study highlight the significant advantage *A. bihariensis* and *A. torrentium* have against the imminent invasion of *F. limosus*. It appears that the aquatic habitat conditions at the limit between lowland and submontane areas act as a decisive factor against the establishment of *F. limosus* populations (also noted by Petrusek et al. 2006; Bonk and Bobrek 2020). Although some studies suggest the potential spread of *F. limosus* into subalpine areas (Garzoli et al. 2020; Boggero et al. 2023), their findings fall within our predicted range for the species (below 370 m asl). The likelihood of high FFP values is low at such altitudes, as it is based on slope gradient and catchment slope.

Invasions may impede native species populations connectivity (Groza et al. 2021), hindering migration between existing populations which may otherwise be apparently safe within protected areas (Pârvulescu et al. 2020). We can easily observe that *A. bihariensis* is relatively safe in this respect, as the separation distances between the predicted invasion boundary and the native species suitable network are quite substantial, indicating that they might be also secure in the near future. However, it is essential not to overlook the possibility of other invasive species' expansion, which could be more aggressive in this respect (e.g.,

Pacifastacus leniusculus, whose presence has been recorded (Weiperth et al. 2020) in neighboring Hungary). As for *A. torrentium*, it is in a more alarming situation, as only 45% of populations are at some distance from the potentially invaded zone, and these distances are smaller compared to *A. bihariensis* (see Table 4). Over half of the populations are already in potential contact, even though field investigations did not find mixed populations with *F. limosus*. This situation has not changed in the past ten years (see Pârvulescu et al. 2012; Pârvulescu et al. 2015; Pacioglu et al. 2020). Further efforts should go towards establishing, monitoring and managing "ark-sites" for the long-term conservation of this native species.

We did not detect *F. limosus* DNA in the qPCR analysis from paths. Still, we cannot exclude the possibility of false negative results given by a low number of crayfish at the marginal sectors of the invasion front. Moreover, the specific environmental conditions in the upstream sectors could be suspected to hinder eDNA detectability (Curtis et al. 2021; Snyder et al. 2023).

Overall, we need to remain cautious, especially considering the potential expansion of crayfish plague pathogen *A. astaci* virulent strains. To the best of our knowledge, this screening represents the most extensive investigation of *A. astaci* prevalence in native crayfish populations. We found an infection rate of 64% in apparently healthy native crayfish populations, with no observed mass mortality events. The rate may be underestimated because of the low amount of tissue used for the qPCR analysis. However, killing healthy, protected crayfish for more reliable results was not an ethical option.

The haplotype found in the three locations with native crayfish species (including *A. astacus* found in a mixed population with *A. torrentium*, see Table 5) was identical in the sequenced fragment of the ribosomal rnnS and rnnL region to sequences of the A-haplogroup (containing the genotype As and Up), a haplogroup with strains ranging from non-virulent to highly virulent (Becking et al. 2015; Boštjančić et al. 2022). While the genotype As has been identified as latent infection in several European crayfish populations (e.g., Viljamaa-Dirks et al. 2013; Jussila et al. 2021), the genotype Up is known from mass mortality in Czechia (Kozubíková et al. 2008; Grandjean et al. 2014). Since *A. astaci* has been present in the Danube and its delta since the first infection wave (Alderman et al. 1987) and is now coexisting with native crayfish (Schrimpf et al. 2012), it is possible that this has also caused the latent infection we found in native populations in this study.

Although the current conditions appear to be stable, this may change in the future. Since *P. leniusculus* is also present in the Danube, but still far from the analysed area at this study date (Weiperth et al. 2020), mortality caused by a more infectious *A. astaci* haplotype is a forthcoming threat, as different vectors other than crayfish may also be responsible for the pathogen's spread (Oidtmann et al. 2002; Águas et al. 2014; Svoboda et al. 2017).

Conclusion

The predictive models enabled the identification and quantification of the degree of proximity between competing species (two native *Austropotamobius* and the invasive *F. limosus*). The expansion of *F. limosus* in streams was found to be limited by flash-flood potential

(a variable measuring stream disturbance potential according to the local and upstream drainage velocity) in a range that is characteristic to lowland rivers. The study revealed *A. bihariensis* is safe against invasion, having large sectors separating it from the invasion front, sectors in which neither *F. limosus* nor the pathogen *A. astaci* was detected. The situation is worrying for *A. torrentium*, as it has many populations at high risk of contact with the invader. A latent infection with *A. astaci* (A-haplogroup) in apparently healthy populations of both species was detected with a low virulent strain, without mass mortality events. Consequently, the conservation efforts in the areal of *A. bihariensis* must focus on preventing the spread of other more virulent crayfish plague pathogen strains, whereas a careful monitoring and management of the ongoing situation of *A. torrentium* is required.

Acknowledgments

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Supplementary material 1

Hyperparameter tuning using Grid Search
Authors: Alina Satmari, Kristian Miok, Mihaela C. Ion, Claudia Zaharia, Anne Schrimpf, Lucian Pârvulescu
Data type: docx
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Supplementary material 2

Results of two samples Welch t-tests for comparisons between species with respect to the geospatial variables in occurrence sites
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Supplementary material 3

Overview of the results from the eDNA analysis for the detection of
A. bihariensis/_A. torrentium_, _F. limosus_ and _A. astaci_
Authors: Alina Satmari, Kristian Miok, Mihaela C. Ion, Claudia Zaharia, Anne Schrimpf, Lucian Pârvulescu
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REVIEW ARTICLE

Tracing the origins and tracking the movements of invasive rubber vines (*Cryptostegia* spp., Apocynaceae)

Marion K. Seier[1], Alessandro Rapini[2], Kate M. Pollard[1],
Robert W. Barreto[3], Harry C. Evans[1]

1 *CABI UK, Bakeham Lane, Egham, Surrey, TW20 9TY, UK* **2** *Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, Feira de Santana, Bahia, 44036-900, Brazil* **3** *Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570-900, Brazil*

Corresponding author: Marion K. Seier (m.seier@cabi.org)

Abstract

Cryptostegia grandiflora and *C. madagascariensis* (Apocynaceae) are the only two species of this Madagascan plant genus. Both have been transported around the world as ornamentals due to their attractive flowers and based on a perceived potential as sources of rubber – hence, the common name rubber vine – because of their copious latex, which also contains toxic cardiac glycosides. As a result of their vigorous growth and ability to climb over and smother vegetation, both species have become invasive, posing an actual or potential threat to native ecosystems in many tropical and sub-tropical countries, as well as to human and animal health. Classical biological control (CBC), or the introduction of co-evolved natural enemies to control an invasive alien species in its exotic range, has successfully been used to tackle *C. grandiflora* in northern Queensland, Australia. This strategy is currently being evaluated for its suitability to manage *C. madagascariensis* in north-eastern Brazil using the same Madagascan rust fungus, *Maravalia cryptostegiae*, released as a CBC agent in Australia. For CBC to be successful, it is critical to understand the taxonomy of the invader as well as the origin(s) of its weedy biotype(s) in order to select the best-matched co-evolved natural enemies. Based on an exhaustive search in published and unpublished sources, we summarise the taxonomy and uses of these rubber vines, follow their historical movements and track their earliest records and current weed status in more than 80 countries and territories around the world.

Keywords

Introduction

Cryptostegia (Apocynaceae, Periplocoideae) is a plant genus native to Madagascar with two accepted species: *Cryptostegia grandiflora*, commonly referred to as rubber vine and *C. madagascariensis*, alternatively named Madagascar rubber vine (Klackenberg 2001; Rojas-Sandoval and Acevedo-Rodríguez 2013a, 2013b; WFO 2022). These perennial woody vines have showy light-pink or purple-pinkish flowers, respectively and produce a milky poisonous latex containing cardiac glycosides. Being climbers, both species can grow up into adjacent taller vegetation, as seen particularly for *C. grandiflora* in riverine forests in Madagascar, but commonly also grow as sprawling shrubs along creeks and gullies, especially in disturbed habitats (Marohasy and Forster 1991). Due to their attractive appearance, as well as their latex, *C. grandiflora* and *C. madagascariensis* have been introduced as ornamentals and/or as potential sources of rubber into numerous countries around the world. In many of their introduced ranges, both species have subsequently become aggressive invaders; smothering native vegetation and threatening local biodiversity, as well as livelihoods (McFadyen and Harvey 1990; Rodriguez-Estrella et al. 2010; Sousa et al. 2016; Bekele et al. 2019; de Lucena et al. 2021). The extent of such invasions can be vast and include large conservation or environmentally-sensitive areas, rendering conventional methods of control by mechanical and/or chemical means inadequate and uneconomic. In these situations, classical biological control (CBC) – an environmentally benign and sustainable method, based on the use of co-evolved and highly specific natural enemies from the invader's native range for control in its introduced range – can offer a promising alternative method for control or form part of an integrated management strategy. Australia pursued this approach when embarking on a CBC programme to tackle the *C. grandiflora* invasion in tropical Queensland in the 1980s. This biocontrol initiative, based on the use of a rust fungus, is now considered to be one of the most successful ever implemented on this continent (Page and Lacey 2006; Palmer et al. 2010).

In order to achieve such success, it is fundamental to correctly determine the taxonomic position of an invasive plant species, as well as the biotype(s) present in the invaded country or region, in order to achieve a close match with its compatible natural enemies from the native range. This is especially critical when exploiting plant pathogens, such as rust fungi as biocontrol agents; typically, these are host specific at both the inter- and intra-species level. Where multiple or mixed introductions have taken place – particularly commonplace for plant species of horticultural or ornamental interest, such as *Lantana camara* (Verbenaceae) (Thomas et al. 2021) – it is crucial to establish such matches for all the invasive biotypes present. In the pre-molecular era, when field surveys searching for CBC agents in the native range had to rely solely on traditional plant taxonomic skills and herbarium records, identifying the area(s) in the centre of origin harbouring biotypes of the target plant species best-matched with the weed biotype(s) occurring in the invaded exotic range was inherently difficult. This is probably why a number of weed CBC programmes have been viewed as failures or only as partial successes – despite the fact that the natural enemy releases may have contributed to some degree of control of susceptible weed populations – because their

impacts were cryptic and went unnoticed (Hoffman and Moran 2008; Barton 2012; Schwarzländer et al. 2018; Morin 2020).

A prime example of the complexity and problems involved when working with rust biocontrol agents is that of the invasive skeleton weed, *Chondrilla juncea* (Asteraceae), in Australia and its co-evolved rust, *Puccinia chondrillina* (Pucciniaceae), from the centre of origin in the Mediterranean Region. Following the initial release of a rust strain from Italy, populations of skeleton weed fell dramatically and this success has been well documented (Cullen et al. 1973; Burdon et al. 1981). However, the introduced rust strain or pathotype proved to be so specific that unrecorded resistant plant biotypes came to the fore and replaced the previously dominant rust-susceptible populations. Using isoenzyme techniques for biotype-pathotype matching, additional rust strains from both Italy and Turkey were released to achieve control of the emergent weed populations (Cullen and Hasan 1988). Similarly, the previously mentioned Australian biocontrol initiative against *C. grandiflora* became successful only following the release of a second strain of the host-specific Madagascan rust *Maravalia cryptostegiae*. Initial releases, undertaken with a strain sourced from *C. madagascariensis* in the northern region of Madagascar, proved to be ineffective against the congeneric target weed (Evans and Tomley 1996). In more recent times, molecular techniques have been adopted for CBC to better identify centres of origin of invasive alien plant species, as well as to pinpoint specific biotypes, thereby improving the chances of finding better-matched, co-evolved natural enemies. For example, a molecular analysis has been used recently to identify the biotypes of Himalayan balsam (*Impatiens glandulifera*, Balsaminaceae), an invasive weed in the British Isles, based on chloroplast DNA sequences (Kurose et al. 2020). The results indicated that at least three separate introductions of this ornamental plant were made and that those biotypes resistant to the two strains of a rust *Puccinia komarovii* var. *glanduliferae*, from north-west Pakistan and north-west India, released in the UK thus far, probably originated in the eastern Kashmir Region of the Himalayas. Further targeted surveys to collect and identify additional rust strains from this region have been initiated to address the problem.

Currently, a similar study is underway as part of a CBC project for Brazil aiming to match pathotypes of *M. cryptostegiae*, under evaluation as a biocontrol agent, with the biotype(s) of *C. madagascariensis* invading the north-eastern region of the country. Literature searches to establish the identity of these weed populations and to trace their origin have revealed a complex history of inter-continental transport of *Cryptostegia* species spanning centuries. In addition to summarising the taxonomic debate surrounding the genus *Cryptostegia*, we track the movements linked to its uses and assess the environmental impact of the two rubber vines from Madagascar in the countries and regions where they have been introduced.

Taxonomic history

The genus *Cryptostegia* was erected to accommodate the single species *C. grandiflora*, based on a specimen sent to the Royal Botanic Gardens (RBG) Kew from a hot-house

plant cultivated in the English Home Counties: "where it flowered in summer, we believe, for the first time in Europe" (Brown 1820). Robert Brown was the botanical consultant at RBG Kew and Keeper of Botany at the British Museum (Desmond 1995) and "The name [*Cryptostegia*] was suggested to Mr. Brown by the circumstances of the enclosure of the five-scaled crown within the tube of the corolla and it not being exposed to view as in other bordering genera" (Brown 1820). In the absence of a holotype, the illustration of this specimen (Brown 1820: t. 435; see Fig. 1) was chosen by Marohasy and Forster (1991) as the lectotype.

The main description of *C. grandiflora* in Brown (1820) is actually by Roxburgh, under the name *Nerium grandiflorum*, based on a specimen collected in India and listed in Hortus Benghalensis (Roxburgh 1814), but only published posthumously, nearly two decades later, in Flora Indica (Roxburgh and Carey 1832). However, before Roxburgh left India in 1813, he appears to have sent the description – as well as a drawing, listed in Icones Roxburghianae (Sealy 1956; see Fig. 2) – to RBG Kew, which was used to complement Brown's type description of the genus *Cryptostegia*. The latter is brief and in Latin, preceding the body of the paper, which was written by the editors of the *Botanical Register* – a short-lived journal devoted to 'Exotic plants cultivated in British Gardens'. In this case, the exotic *C. grandiflora* had been grown by Sir Abraham Hume – on his estate at Wormleybury, Hertfordshire – who, as a director of the East India Company, maintained a large collection of rare Indian plants regularly sent to him by William Roxburgh from the Calcutta Botanic Garden (Harwood 2007; Kochhar 2013).

According to Roxburgh (1814), the collection of *N. grandiflorum* in the Botanic Garden at Calcutta was sent by Dr B. Heyne from southern India in 1804. Benjamin Heyne was a botanist employed by the East India Company who was variously based at botanical gardens in Bangalore and Mysore (Sikarwar 2020). The fall of the Mysore Sultanate in 1799 opened up access to the Western Ghats and the Malabar Coast, allowing plant collections to be undertaken in these areas (Heyne 1814). In the latter publication, Heyne devotes a section to latex-producing plants and *Nerium* is cited in the list. It is reasonable to suppose, therefore, that Heyne would have labelled the rubber-vine material that he despatched to Roxburgh in 1804 as an unknown and endemic species of *Nerium*. All the subsequent references quote *C. grandiflora* as being "A native of the Peninsula of India" (Brown 1820; Roxburgh and Carey 1832). This raises the further assumption that *C. grandiflora* had been present in south-west India for a considerable period of time, becoming naturalised and accepted locally as part of the native flora and not as an exotic species. There are several overriding questions: why did rubber vine not become invasive in the region; and who introduced it from Madagascar and when?

Historical events point to the Portuguese who colonised the area around Cochin on the Malabar Coast in the early 16th century and later established a viceroy ship there. Around this period, the first attempt at European colonisation of Madagascar was also by the Portuguese; although the first permanent settlement was not established until around 1615 at the behest of the Portuguese Viceroy of India. This colony in southern Madagascar, near Fort Dauphin (Taolagnaro), became pivotal in the transoceanic trade route between Portugal and India (Brown 2001). Plants from Portuguese

Figure 1. Lectotype of *Cryptostegia grandiflora* in Brown (1820), based on a flowering specimen donated by Sir Abraham Hume from his hot-house at Wormleybury Manor, Hertfordshire, England.

colonies in Africa and the Americas (Brazil) and, presumably, also from Madagascar, arrived in India via this route (Gavali and Lakhmapurkar 2018; Sikarwar 2020). *Cryptostegia grandiflora* is a common plant in the southern region of Madagascar (Marohasy and Forster 1991; Klackenberg 2001) and, thus, may have attracted the attention of the Portuguese colonists, either as an ornamental or for its purported local uses in making cloth and rope for fishing nets (Jumelle 1907; Klackenberg 2001).

The new species *C. madagascariensis*, in Bojer's (1837) Hortus Mauritianus – or, 'the exotic and indigenous plants cultivated in Mauritius' – was the first indication that *Cryptostegia* might not be native to India. Bojer recorded *C. madagascariensis* as present in botanical gardens on Mauritius, but gave its origin as Madagascar, specifically

Figure 2. Illustration of *Cryptostegia grandiflora* (as "*Nerium grandiflorum* Roxb."), from Icones Rox-burghianae (Sealy 1956); drawings commissioned by William Roxburgh of plants in the Calcutta Botani-cal Garden and sent to RBG Kew together with specimens and descriptions, between 1793 and 1813.

as a coastal plant common around the Bay of Bombetok[a], which lies in north-west Madagascar close to the port of Majunga (Mahajanga). His view was reinforced by P. Koenig, a plant collector who sent specimens to Kew from Mauritius in 1907–1908 and who posited that *Cryptostegia* had already been introduced on to the island by the Malagasy people two to three centuries earlier (Klackenberg 2001). Bojer (1837) also listed *C. grandiflora* as growing in Mauritius, but still gave its origin as India. Decaisne (1844) gave a full description of the genus and both the species, *C. grandiflora* and *C. madagascariensis*; the former said to be from India, the latter from Madagascar. Amongst the critical distinguishing characters between the species, he noted the co-rona lobes as being bifid or bilobed in *C. grandiflora* and entire in *C. madagascariensis*, which has since been confirmed by others (Hemsley 1904, see Fig. 3; Jumelle 1908; Polhamus et al. 1934; Marohasy and Forster 1991; Klackenberg 2001). Costantin and Gallaud (1906) listed both *C. grandiflora* (local name: lombiri) and *C. madagascariensis* (local name: lombiro) from Madagascar, but described the former as exotic ("non in-digène") and growing spontaneously. Furthermore, they named an indigenous variety from the Tulear (Toliara) Region, *C. grandiflora* var. *tulearensis* (local name: lombiri-voharoto), distinguished by its smaller, more elongated leaves and smaller fruits (Cos-tantin and Gallaud 1906).

   Subsequently, Jumelle (1908, 1912) confirmed the presence of *C. grandiflora* in Madagascar – specifically, being confined to the southern region and reaching as far as Tulear in the south-west – but was unclear about its origin. He still appears to have included India within its natural range, describing it as common, whilst list-ing it as having been introduced into Mauritius and Réunion, as well as into Egypt,
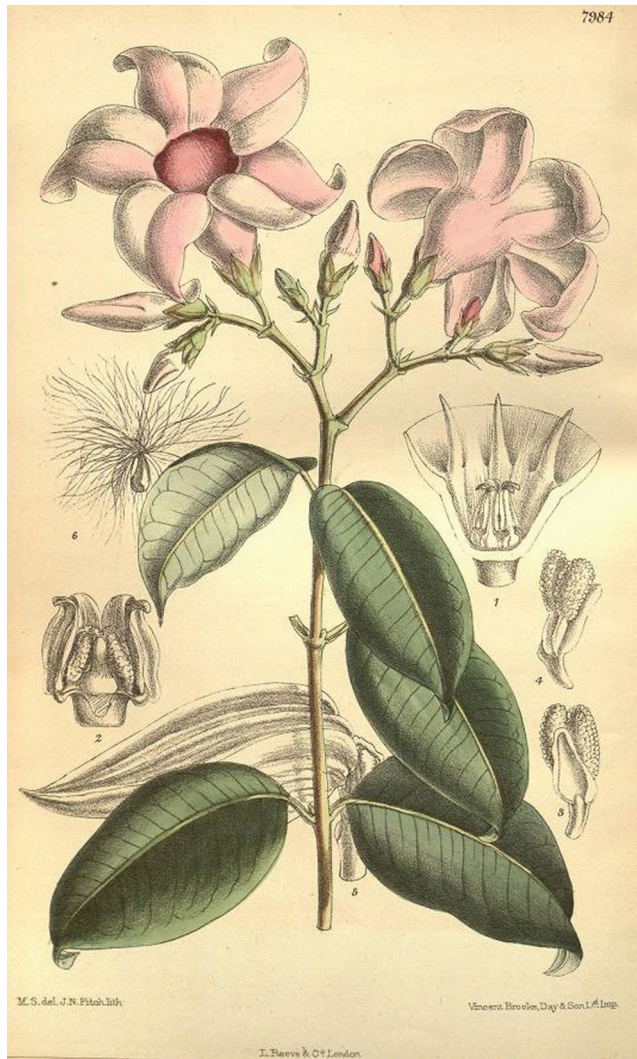
Figure 3. Illustration of *Cryptostegia madagascariensis* in Hemsley (1904), drawn from a plant grown at Royal Botanic Gardens, Kew.

Sudan, Java, Mexico and Cuba. Klackenberg (2001) considered that Jumelle (1908) was, in fact, the first to recognise that *C. grandiflora* is a Madagascan endemic – and, therefore, that it must have been introduced into India – but this interpretation is open to question and, from the literature review, it still remains unclear exactly when *C. grandiflora* was confirmed definitively as being indigenous to Madagascar and exotic in India. As late as 1975, it was still being described as native to India, whilst the Madagascan endemicity of *C. madagascariensis* was unambiguous (Spellman 1975). Nowadays, the Madagascan origin of both *Cryptostegia* species is undisputed (Ionta and Judd 2007).

Taxonomic status

The two species recognised in the most recent treatment of the genus (Klackenberg 2001) – *C. grandiflora* and *C. madagascariensis* – are morphologically separated as follows:

"Calyx lobes > 13 mm long; corona lobes bifid; spathe of translator orbicular, obtuse at apex; leaves always glabrous; follicles often more than 10 cm long.................... .... **1. *C. grandiflora***
Calyx lobes ≤ 13 mm long; corona lobes entire; spathe of translator ovate, acute at apex; leaves sometimes hairy; follicles shorter than 10 cm............**2. *C. madagascariensis***"

The flower main characteristics to separate the two species were illustrated by Curtis (1946; see Fig. 4).

Hochreutiner (1908) distinguished *Cryptostegia glaberrima* from *C. madagascariensis* using the lack of leaf indumentum as one criterion, while Marohasy and Forster (1991) delimited three varieties of *C. madagascariensis*: var. *madagascariensis*, with sparse to dense indumentum on both leaf surfaces; var. *glaberrima*, glabrous on both surfaces; and var. *septentrionalis*, with indumentum only on the upper surface. However, Klackenberg (2001) considered that this is "a taxonomically useless character in *Cryptostegia*", since he encountered varying degrees of leaf hairiness in the continuum of populations of *C. madagascariensis* along the west coast of Madagascar. While not having seen the type of *C. grandiflora* var. *tulearensis*, Klackenberg (2001) considered this as an uncertain taxon and likely a synonym of *C. grandiflora*.

Distribution and ecology in Madagascar

Marohasy and Forster (1991) were the first to map and interpret the distribution and ecology of the genus *Cryptostegia* in Madagascar, following extensive surveys from 1987–1988 by the former author. *Cryptostegia madagascariensis* was found to have a continuous distribution along the western coast, from Tulear in the south-west to Maromandia in the north-west, a distance of some 1600 km, occurring naturally in riverine and seasonally-flooded forests in areas with an annual rainfall between 600 and 1800 mm. However, they noted that it could be an aggressive invader in disturbed habitats and especially in secondary savannahs, where it grows in full sun (Fig. 5A). The geographically-isolated populations in the extreme north-west region around Diego Suarez (Antsiranana), with a monsoonal climate (1200–1800 mm per annum) – separated taxonomically as var. *septentrionalis* (Marohasy and Forster 1991) – occur in gullies and often form dense stands in coastal saltmarshes (Fig. 5B). They seem to have a distinct ecological niche and are possibly genetically isolated. Differences in populations like these may be relevant and could explain their ability (or not) to invade exotic ecosystems and, ultimately and critically, define the success of any biological control programme by using best-matched natural enemies.
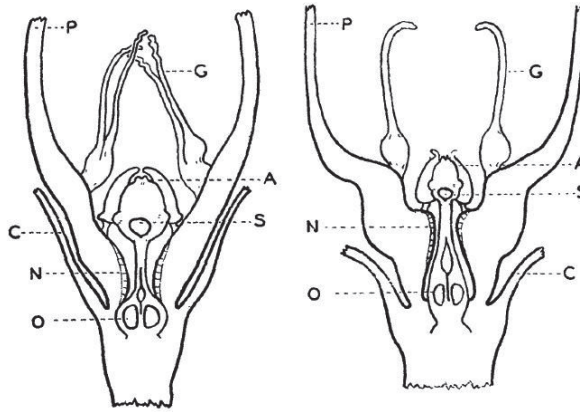
Figure 4. Diagram of a longitudinal section of *Cryptostegia grandiflora* (left) and *C. madagascariensis* (right) flowers; showing the corolline corona (G) in *C. grandiflora*, with bifid lobes converging at the tips and hiding the anthers (A) and stigma (S) – hence the generic descriptor – whilst those of *C. madagascariensis* are entire and separate. C = calyx, N = nectary, O = ovary, P = corolla lobes; ex Curtis (1946).
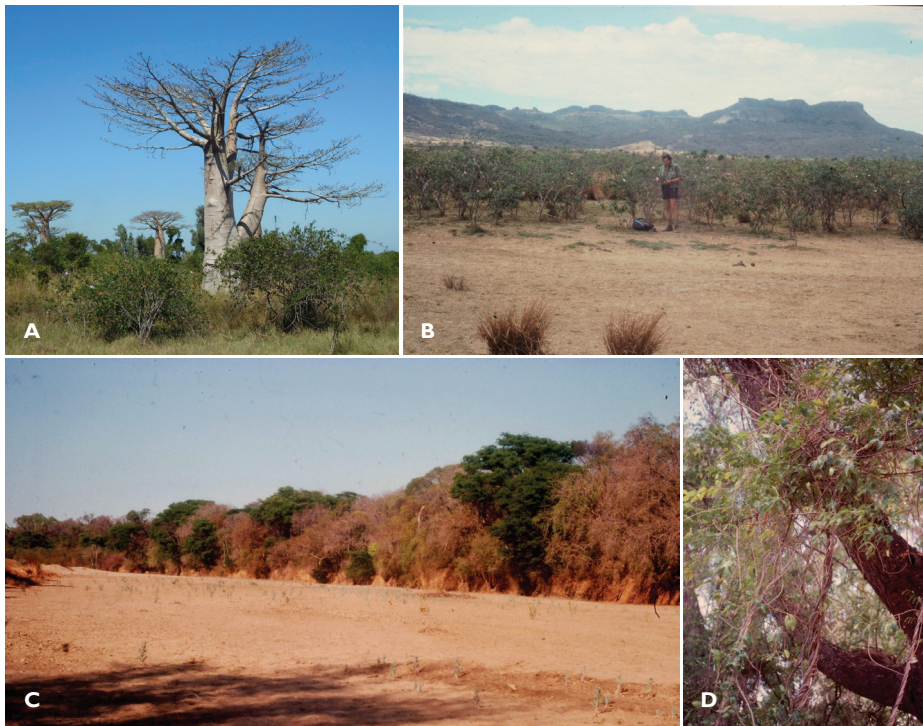
Figure 5. Habitats of *Cryptostegia* in Madagascar **A** *C. madagascariensis* forming low shrubs in savannah with typical baobab vegetation, Morondava-Manja area, west-central Madagascar **B** *C. madagascariensis*, in littoral locality forming dense, low stands on compacted sand, Ramena beach, Diego Suarez, northern Madagascar **C** gallery-forest habitat of *C. grandiflora* in south-west Madagascar, along dry river bed **D** lianas of *C. grandiflora* growing into the canopy of tamarind trees within gallery forest, Betioky, south-west Madagascar.

Cryptostegia grandiflora has a narrower distribution in Madagascar, being restricted to the dry south-west region, some 600 km distance between Tulear and Fort Dauphin, with an annual rainfall of less than 600 mm. It is a vigorous climber in gallery or riverine forests (Fig. 5C, D) and common in disturbed habitats, occurring along river beds, creeks and gullies, from sea level to ca. 500 m.a.s.l. The two species are sympatric between the Fiherenana and Onilahy Rivers, north of Tulear, from where putative hybrids have been reported (Marohasy and Forster 1991).

Uses

Morais et al. (2021) reviewed the plant chemistry of the genus *Cryptostegia* in relation to its biological activities and industrial applications. Here, we examine more critically the attempts to utilise the genus as a crop and as a source of medicinal products.

Rubber

Danthu et al. (2016) charted the history of rubber production in Madagascar, which was based on crude extractions from the logged stems of various members of the Euphorbiaceae and Apocynaceae. They included both species of *Cryptostegia*, in which the quality of rubber from *C. grandiflora* had previously been noted by Jumelle (1912) as being superior to that from *C. madagascariensis*. The use of latex, or gum elastic, by the indigenous peoples had been recorded as long ago as the end of the 18th century (Danthu et al. 2016). However, it was not until the latter part of the 19th century that exports of rubber to Europe commenced and by the end of this and the early 20th century that it formed the major export from Madagascar. Palay rubber – as the product from *Cryptostegia* was known on the international commodity market, although this name has its origin in India and is probably derived from the Tamil word, palai – occupied around 20–30% of the total rubber exports; the greater percentage coming from the Euphorbias (Danthu et al. 2016). As these authors highlighted, this came at great cost to the different forest ecosystems from where the plants were extracted and the increasing scarcity of raw material plus the rise of the superior *Hevea* rubber led to the decline of rubber production in Madagascar after the First World War. However, during the Second World War – as Madagascar passed into Allied hands – there was a concerted, but short-lived effort (1943–1945) to resuscitate the rubber industry in order to secure the world demand following the Japanese occupation of the *Hevea* plantations in Asia (Danthu et al. 2016).

These events also led the USA to invest more heavily in alternative sources of rubber, including *Cryptostegia*, through the Emergency Rubber Project. Experimental plantations were initiated or revived in various tropical countries of the Americas, especially in Haiti (Knight 1944; Finlay 2009). Based on previous post-war research, Palay rubber was considered to be equivalent or even superior to *Hevea* rubber and high-yielding hybrids had been developed in the USA (Polhamus et al. 1934). This *Cryptostegia* material formed the basis of the multi-million dollar rubber project

initiated in Haiti in 1943, with over 60,000 acres being commandeered and cleared for planting (Symontowne 1943). However, a combination of various factors hampered the initiative, including: devastating insect attacks (Knight 1944), drought, difficulties in harvesting and, more critically, poor yields due to the technical problems involved with latex extraction (Stanton 1944). This resulted in "The embarrassing collapse of *Cryptostegia*" (Finlay 2009), with serious socio-economic and political ramifications in both Haiti and the USA. These historical failings with rubber-vine cultivation were not addressed by Augustus et al. (2000) who explored the potential of *C. grandiflora* as a multi-purpose crop in India, particularly as an alternative source of biofuel. They noted that "It grows profusely without agronomic management": one of the reasons that it failed in Haiti was because of harvesting logistics (Finlay 2009) and, of course, a trait that makes rubber vine such an aggressive weedy invader.

Fibre

Jumelle (1907) gave a detailed account of the production of fibre from *C. madagascariensis* in north-west Madagascar, which was used to make textiles, rope and fishing nets. However, there are no reports of its current exploitation as a fibre crop in Madagascar or elsewhere, except perhaps for Papua New Guinea (fide Herb K annotation).

Medicinal

There are various reports in the literature of the anti-tumour and anti-microbial potential of bioactive extracts of *C. grandiflora*, as well as analgaesic properties (Doskotch et al. 1972; Mukherjee et al. 1999; El Zalabani et al. 2003; Singh et al. 2011; Hanumanthappa et al. 2012; Morais et al. 2021). Castro et al. (2014) reported that *C. grandiflora* was widely used in folk medicine on the Caribbean coast of Colombia – particularly, as an anti-inflammatory – and identified metabolites in the leaves that proved to have anti-inflammatory properties in both *in vitro* and *in vivo* experiments. Similar ethnobotanical surveys in India also revealed that leaf decoctions of *C. grandiflora* were used to treat liver and nervous disorders (Wagh and Jain 2018).

Conversely and somewhat ironically, in their Madagascan centres of origin, the two rubber-vine species have limited medicinal uses, although Jumelle (1907) did note that the Sakalava tribe of western Madagascar prepared root decoctions of *C. madagascariensis* to cure chronic gonorrhoea. In fact, these vines are avoided by most ethnic groups who often warn travellers about the toxic dangers of the latex (Evans HC, pers. obs. 1988) – which contains glycosides that affect cardiac systems – and *C. grandiflora* has been linked with both animal and human deaths in Australia (McFadyen and Harvey 1990). In Madagascar, *C. madagascariensis* has been traditionally used as a poison to commit suicide or against enemies (Jumelle 1907), especially by 'wronged' wives. Ironically, in its exotic range, it is also known as 'flor de muerto' (death flower) in Colombia (Castro et al. 2014) and as 'viuvinha' (small widow) or 'viúva-alegre' (happy widow) in north-east Brazil (Sousa et al. 2016; Morais et al. 2021).

Weed status

This section covers those continents or geographic regions for which data regarding the presence and status of the two *Cryptostegia* species exist. Data were gathered from both published and unpublished sources, i.e. reports and herbaria records, as well as from web-based sources: namely, the Global Biodiversity Information Facility (GBIF), the Germplasm Resources Information Network (GRIN) and the databases Tropicos, the Global Invasive Species Database (GISD), Pacific Islands Ecosystems at Risk (PIER) and Plants of the World online (POWO). All herbaria consulted are referred to with their acronyms according to Thiers (2022). Identification of individual *Cryptostegia* species from herbarium specimens could not always be confirmed, as online images were often not available or specimens were not well preserved. Thus, in most cases, the species identification given on the respective labels and in the databases was accepted. Table 1 lists occurrences of *C. grandiflora* and *C. madagascariensis* for individual countries and territories with earliest records, where known and additional information. Those for which more detailed information is available – concerning the history of introduction, the use and current status of the species – are discussed below. The situation in Australia is treated in depth because it is where *Cryptostegia* was first identified as a major invasive weed and a management strategy for its control was pioneered.

Asia

China (Hong Kong)

A specimen of *C. madagascariensis* in Herb K from the Hong Kong Botanical Garden dated 1879 indicates that the species must have been introduced from another British colonial botanical garden.

India

The history of *C. grandiflora* in India has already been discussed at length. Suffice to say that there are few publications of it as a problematic or invasive weed. In the invasive alien flora of India (Srivastava et al. 2014), *C. grandiflora* is described as an "aggressive coloniser" and "occasional in forests", but quantitative data are lacking. It is also listed in the invasive alien species of Uttar Pradesh – with its weedy status described as "interfering" – but it is not included in the list of India's most noxious weeds (Reddy et al. 2008). Similarly, it is included under its native name, 'rubber-bel', in a study of the plant composition of a conservation area in Rajasthan, but with no indication of its invasive or alien status in the comments on invasive alien weeds (Chaudhary and Shringi 2017). The earliest Herb K record is from January 1804, labelled "*Echites-Apocynum*, from the Governor's garden" (Fig. 6). Exactly from which city or region is unknown, but this coincides with the date when a collection labelled *N. grandiflorum* was sent from southern India to the Botanic Garden at Calcutta, as discussed earlier. The oldest specimen of *C. grandiflora* for which a locality is documented was collected by Herbert Wight in the southern State of Tamil

Table 1. Occurrences, earliest records and current status of *Cryptostegia grandiflora* and *C. madagascariensis* by individual countries.

Country/ Region	*Cryptostegia grandiflora*			*Cryptostegia madagascariensis*			References[c]
	Presence[a]	Earliest record[b]	Notes	Presence	Earliest record	Notes	
Asia							
Bangladesh	+	u	–	-	–	–	POWO (2022)
China: Hong Kong	-	–	–	+	1879 (Herb K)	–	Herbarium record
India	+	1804 (Herb K)	cultivated, established	+	u	established	Chaudhary and Shringi (2017); POWO (2022); Srivastava et al. (2014)
Indonesia	+	1897 (Herbs LD, UPS)	cultivated, not naturalised	-	–	–	Setyawati et al. (2015)
Pakistan	+	1962 (Herb SINDH)	cultivated	-	–	–	Flora of Pakistan (2022)
Philippines	+	u	cultivated, Merrill (1912) states introduction as recent	+	1955 (Herb US)	garden record	Herbarium record (*C. madagascariensis*); Merrill (1912); Razon (2008)
Saudi Arabia	+	1893 (Herb L)	–	-	–	–	Herbarium record
Singapore	+	u	cultivated	-	–	–	Chong et al. (2009)
Taiwan	-	–	–	+	1971 (Herb TAI)	–	GBIF (2021)
Yemen	+	–	cultivated	-	–	–	Alasbahi and Al-Hawshabi (2021)
Africa							
Angola	+	–	as *Cryptostegia* sp. in Herb LISU, Herbario Angola	+	–	as *Cryptostegia* sp. in Herb LISU, Herbario Angola	GBIF (2021)
Botswana	+	u	naturalised	-	–	–	Witt and Beale (2018)
Burkina Faso	+	u	–	-	–	–	POWO (2022)
Comoros	+	u	–	-	–	–	POWO (2022)
Congo	-	–	–	+	u	–	POWO (2022)
Cote d'Ivoire	+	1995 (Herb UCJ)	–	-	–	–	GBIF (2021)
Egypt	+	1904 (Herbs S, UPS)	cultivated	-	–	–	El Zalabani et al. (2003)
Ethiopia	+	1972 (Herbs MO, WAG in L)	invasive in the Afar and Shewa regions	-	–	–	Bekele et al. (2019); Luizza et al. (2016); Witt and Luke (2017); Witt et al. (2018)
Gambia	+	u	–	-	–	–	POWO (2022)
Ghana	+	1927 (Herb GC)	–	+	1932 (Herb K)	–	Asase (2021); Herbarium record (*C. madagascariensis*)
Guinea	+	u	–	-	–	–	POWO (2022)
Kenya	+	u (Herb US)	–	+	1970 (Herb K)	cultivated, established, record of the rust *M. cryptostegiae* from 1950	Herbarium record (*C. grandiflora*); Witt and Beale (2018); Witt and Luke (2017)
Madagascar	+	1879 (Herb MO)	endemic	+	1911 (Herbs MO, S)	endemic	Costantin and Gallaud (1906); Jumelle (1908, 1912); Klackenberg (2001); Marohasy and Forster (1991)
Malawi	-	–	–	+	–	naturalised	Witt and Beale (2018)

Country/Region	*Cryptogegia grandiflora*			*Cryptostegia madagascariensis*			References[c]
	Presence[a]	Earliest record[b]	Notes	Presence	Earliest record	Notes	
Mali	+	u	–	-	–	–	POWO (2022)
Mauritius	+	1867 (Herb K)	record from Hooker herbarium at Herb K, established	+	1867 (Herb K)	specimen from Hooker herbarium at Herb K, naturalised, recorded as native in GISD and PIER (referencing outdated version of GRIN)	Bojer (1837); GRIN (2022)
Mayotte	+	u	cultivated	+	–	undated record in Herb P	GISD (2022); Herbarium record (*C. madagascariensis*); PIER (2022)
Morocco	+	u	–	-	–	–	Rojas-Sandoval and Acevedo-Rodríguez (2013a)
Mozambique	+	u	–	+	u	–	POWO (2022)
Namibia	+	1958 (Herbs MO, US)	–	-	–	–	Brain and Fox (1994); Ranwashe (2022)
Nigeria	+	1966 (Herb WAG in L)	–	-	–	–	Herbarium record
Réunion	+	u	naturalised, potentially invasive	-	–	–	Comité Français de L' UICN (2022); Groupe Espèces Invasives de La Réunion (2022)
Senegal	+	1960 (Herb IFAN)	–	-	–	–	GBIF (2021); POWO (2022)
Seychelles	+	u	–	+	u	naturalised, recorded as native in GISD	GRIN (2022); POWO (2022); Robertson and Todd (1983)
Somalia	-	–	–	+	1989 (Herb UPS)	–	Thulin (2006)
South Africa	+	1943 (Herb K)	invasive in Limpopo, Mpumalanga and North–West Provinces	+	1860s (Bot. Garden Cape Town)	invasive in Limpopo and North–West Provinces	Invasives South Africa (2022); Sztab and Henderson (2015a, b)
Tanzania	+	u	–	+	1929 (Herb EA)	cultivated	Witt and Beale (2018); Witt and Luke (2017); Witt et al. (2018)
Zambia	+	u	naturalised	+	–	naturalised	Witt and Beale (2018); Witt and Luke (2017)
Zimbabwe	+	1976 (Herb K)	cultivated	+	–	–	POWO (2022)
North America							
Mexico	+	1897 (Herb US)	invasive in Baja California, Chiapas, Tabasco, Yucatan	+	1930 (Herb NY)	recorded from Baja California, Tabasco, Yucatan	Davidse et al. (2009); Patterson and Nesom (2009); Rodriguez-Estrella et al. (2010); Rojas-Sandoval and Acevedo-Rodríguez (2013a, b)
USA	+	Mainland 1905 (Herb NY), Hawaii 1930 (Herb BISH)	mainland record from New York Botanical Garden, possibly invasive in Texas, Florida	+	Mainland 1905 (Herb NY), Hawaii 1906 (Herb BISH)	mainland record from New York Botanical Garden, invasive in Florida, Hawaii	Patterson and Nesom (2009); Polhamus et al. (1934); Witt and Luke (2017)

Country/ Region	*Cryptostegia grandiflora*			*Cryptostegia madagascariensis*			References[c]
	Presence[a]	Earliest record[b]	Notes	Presence	Earliest record	Notes	
Central America and the Caribbean							
Anguilla	-	–	–	+	u	cultivated	Varnham (2006)
Bahamas	+	1904 (Herb NY)	escape from cultivation	-	–	–	Britton and Millspaugh (1920)
Barbados	+	1906 (Herb US)	–	+	2007 (Herb US)	–	Orrell (2022)
Belize	+	1990 (Herb NY)	–	+	1970 (Herb MO)	cultivated	Balick et al. (2000)
Bermuda	+	u	escape from cultivation	-	–	–	Britton (1918)
British Virgin Isl[d]	+	u	recorded on Tortola Isl	+	u	invasive on Anegada Isl	GRIN (2022); McGowan et al. (2006)
Cayman Isl	+	u	cultivated, naturalised on all three islands	-	–	–	Varnham (2006)
Costa Rica	-	–	–	+	1966 (Herb MO)	Genus recorded at USDA Rubber Station since 1947 (Herb MO), but not identified at species level	Davidse et al. (2009)
Cuba	+	1895 (Herb NY)	invasive	+	1926 (Herb US)	–	González-Torres et al. (2012); Orrell (2022)
Dominica	-	–	–	+	u	–	GRIN (2022)
Dominican Republic	+	1910 (Herb US)	–	+	1977 (Herb MO)	–	POWO (2022)
El Salvador	+	u	–	+	1922 (Herb US)	–	Davidse et al. (2009)
Grenada	-	–	–	+	1924 (Herb US)	–	Orrell (2022)
Guadeloupe	+	1893 (Herb NY)	cultivated in Jardin botanique de la Basse–Terre	-	–	–	Herbarium record
Guatemala	+	2002 (Herb UVAL)	–	+	1994 (Herb UVAL)	–	Universidad del Valle de Guatemala (2022)
Haiti	+	u	not naturalised	+	1927 (Herb US)	not naturalised, identified as *C. grandiflora* in Herbs K and NY	Finlay (2009); Knight (1944); POWO (2022)
Honduras	+	1945 (Herbs F, MO)	invasive	+	1947 (Herb F)	–	Davidse et al. (2009)
Jamaica	+	1858 (Herb K)	not naturalised	+	u	–	POWO (2022)
Martinique	+	u	–	+	early 1900s	naturalised	Courty and Lasalle (2020)
Montserrat	+	1979 (Herb NY)	invasive	+	u	escape from cultivation, invasive	Varnham (2006); Young (2008)
Netherland Antilles	+	Curaçao 1913 (Herb US)	invasive on Curaçao, Aruba, Bonaire, naturalised on Saba, St Maarten/ St Martin, established on St Eustatius	+	Saba 2006 (Herb NY)	recorded on Saba, St Eustatius	Burg et al. (2012); Kairo et al. (2003); Mayfield-Meyer and Zhuang (2022)
Nicaragua	+	1987 (Herb WAG in L)	–	+	1923 (Herb MO)	–	Davidse et al. (2009); Herbarium record (*C. madagascariensis*)

Country/ Region	*Cryptostegia grandiflora*			*Cryptostegia madagascariensis*			References[c]
	Presence[a]	Earliest record[b]	Notes	Presence	Earliest record	Notes	
Panama	+	1977 (Herbs MEXU, MO)	recorded in Darién Province	+	1935 (Herb MO)	recorded in several provinces	Correa et al. (2004); Davidse et al. (2009)
Puerto Rico	+	1913 (Herb NY)	–	+	1915 (Herb US)	naturalised	Acevedo-Rodriguez (2005); Gann et al. (2022); Witt and Luke (2017)
St Lucia	+	1909 (Herb L)	–	+	u	cultivated, naturalised	Graveson (2021); Herbarium record (*C. grandiflora*)
Trinidad & Tobago	+	1909 (Herb L)	–	+	1933 (Herb MO)	–	Powo (2022)
US Virgin Isl	+	1923 (Herb NY)	herbarium record from St Croix, erroneously reported from St John	+	1970 (Herb MO)	naturalised on St Croix, St John, St Thomas	Acevedo-Rodriguez (1996); Acevedo-Rodriguez (2005); Orrell (2022)
South America							
Brazil	+	1906 as *C.* sp. (Herb P), 1916 as *C. grand.* (Herbs SP, IPA)	naturalised in Bahia, Mato Grosso do Sul, Pará, Piauí, Rio Grande do Norte	+	1906 as *C.* sp. (Herb P), 1930 as *C. madagascariensis* (Herb US)	invasive in Ceará, Pernambuco, Piauí, Maranhão, Rio Grande do Norte, recorded in Bahia	da Silva et al. (2008); Flora e Funga do Brasil (2022); Silva et al. (2018)
Colombia	+	1906 (Herb US)	–	+	1899 (Herb US)	–	Gracia et al. (2019)
Ecuador	+	1926 (Herb US)	herbarium record from mainland Bahia, cultivated on Santa Cruz Isl., Galapagos	-	–	–	Guézou et al. (2010)
French Guiana	+	1977 (Herb WAG in L)	–	+	2000 (Herb US)	–	Herbaria records
Guyana	-	–	–	+	1988 (Herb US)	escape from cultivation	Funk at al. (2007)
Suriname	+	1972 (Herb U in L)	–	-	–	–	Herbarium record
Peru	+	1959 (Herb F)	naturalised in Cajamarca	-	–	–	Herbarium record; GRIN (2022)
Venezuela	+	1922 (Herb US)	–	+	1939 (Herb US)	escape from cultivation, recorded in Amazonas, Aragua, Bolívar, Lara, Nueva Esparta, Sucre	Funk et al. (2007); Hokche et al. (2008); Herbarium record (*C. grandiflora*)
Oceania							
Australia	+	1875 (GISD)	invasive in Queensland, recorded in Northern Territory, Western Australia	+	1953 (Australia Virtual Herb)	naturalised, recorded in Queensland, Northern Territory, Western Australia	Atlas of Living Australia (2023a, b); Marohasy and Forster (1991); Tomley (1995)
Cook Isl	-	–	–	+	1993 (Herb CHR)	established, recorded from Isl of Rarotonga, Mangaia, 'Atiu, Penrhyn	McCormack (2007)
Fiji	+	u	established	-	–	–	Meyer (2000)
French Polynesia	+	1831 (Herb BISH)	cultivated on several Isl	+	1926 (Herb MO)–	treated as *C. grandiflora* in Herbs L, MIN, BISH	Florence et al. (2013); Herbarium record (*C. madagascariensis*)
Guam	+	1963 (Herb US)	cultivated, established	-	–	–	Fosberg et al. (1979)

Country/ Region	*Cryptostegia grandiflora*			*Cryptostegia madagascariensis*			References[c]
	Presence[a]	Earliest record[b]	Notes	Presence	Earliest record	Notes	
Marshall Isl	+	1965 (Herb BISH)	recorded on Kwajelein Atoll	-	–	–	Fosberg et al. (1979)
New Caledonia	+	1950 (Herb P)	invasive on New Caledonia Isl, Ile Grande Terre	-	–	–	Meyer (2000)
Northern Mariana Isl	+	u	recorded on Saipan Isl, established	-	–	–	Fosberg et al. (1979)
Palau	-	–	–	+	u	cultivated on Babeldaob, recorded on Koror	Space et al. (2003)
Papua New Guinea	+	1936 As *C.* sp. (Herb K)	cultivated on Bismarck Archipelago	-	–	–	Herbarium record; Peekel (1984)

[a]Presence: + = present, - = not recorded
[b]Earliest record: u = unknown, Herb (herbarium) acronyms according to Thiers (2022)
[c]References. source databases used, quoted where exclusive reference for *C. grandiflora* and/or *C. madagascariensis*: GBIF=Global Biodiversity Information Facility; GISD=Global Invasive Species Database; GRIN=Germplasm Resources Information Network, USDA; PIER=US Forest Service, Pacific Island Ecosystems at Risk; POWO=Plants of the World, Kew Science, UK; Tropicos=Botanical Database Missouri Botanical Garden, USA; full references given in manuscript
[d]Isl = island/islands

Figure 6. Herbarium specimen of *C. grandiflora* from India deposited at the Royal Botanic Gardens Kew (Herb K), collected in 1804 and originally identified as a species of *Echites* or *Apocynacum*; making it the earliest recorded collection of the genus *Cryptostegia*.

Nadu in 1849 (Herb S). *Cryptostegia madagascariensis* is also reported as established in India, but without information when it was first recorded (GISD 2022; POWO 2022).

Indonesia

The first herbarium specimen of *C. grandiflora* dates from 1904 (Herb L), although records document that the species has been present at Bogor Botanical Garden, Java since at least 1897 (Herbs LD, UPS). While *C. grandiflora* is included in a guide book to the invasive alien plants of Indonesia (Setyawati et al. 2015), it is described as rare and an ornamental with no indication that it has become naturalised.

Pakistan

Whilst the earliest herbarium specimen of *C. grandiflora* from Pakistan dates from 1962 (Herb SINDH), a new fungus, *Pleosphaeropsis* (now *Aplosporella*) *cryptostegiae*, was described from dead twigs of rubber vine, collected in 1939 from Lahore – then part of India (Chona and Munjal 1950). Presumably, this was from a cultivated plant and there are no reports of *C. grandiflora* as an invasive species. According to Index Fungorum (2023), there are 12 confirmed fungal taxa bearing the species epithet '*cryptostegiae*', three of which are from the Lahore Region of Pakistan and six from India, all on *C. grandiflora*, as well as two from Brazil. Ironically, there is only a single validated species with this epithet, the rust *M. cryptostegiae*, listed in the Index from Madagascar. This would give the impression that *C. grandiflora* is, indeed, native to the Indian subcontinent: in reality, however, it is more a reflection of the historical dearth of mycologists in Madagascar compared to both India and Brazil.

Yemen

Alasbahi and Al-Hawshabi (2021) reported *C. grandiflora* as present throughout Yemen and cultivated as an ornamental. Its poisonous properties were highlighted, but with no mention of the plant being invasive.

Africa

Egypt

There are no indications that *C. grandiflora* is weedy in Egypt (El Zalabani et al. 2003), although herbarium records in Herbs S and UPS show that it has been cultivated in Cairo since at least 1904.

Ethiopia

Witt et al. (2018) reported *C. grandiflora* as being problematic and invasive in the Awash National Park: "smothering native *Acacia* species and displacing valuable forage

species"; which is based on an earlier study in the Afar Region of north-eastern Ethiopia (Luizza et al. 2016). This was subsequently reinforced by Bekele et al. (2019), who undertook an impact assessment study in the East Shewa Zone of the Oromia Region and concluded that *C. grandiflora* is a major driver of biodiversity loss, as well as posing a threat to the agro-economy because of its impact on pastoralists. First reported in 1972 (Herbs MO, WAG in L), records in Herb K from Ethiopia dating from the same time describe *C. grandiflora* as "rare" to "quite frequent" in the Awash area of the central Highlands, giving no indication that it was problematic or invasive at that time.

Ghana

The presence of *Cryptostegia* was first reported as *C. grandiflora* from Accra in 1927 (Asase 2021), whilst Herb K has a record of *C. madagascariensis* from 1932 with the annotation: "Introduced from Victoria Botanical Gardens" – presumably, in nearby Cameroon, now Limbe Botanical Gardens. There is no indication that either species has become naturalised or invasive in any of the West African countries.

Kenya

Witt and Luke (2017) stated that *C. madagascariensis* is a garden ornamental which has not naturalised in Kenya and Herb K collections from the early 1970s show *C. madagascariensis* being cultivated as an ornamental along the coast, north of Mombasa. However, there is a record in the fungarium of the Imperial Mycological Institute (IMI, now held at RBG Kew) of the rust *M. cryptostegiae* from the same area dating from 1950, with more records of heavily-rusted plants from the 1970s. It is tempting to speculate that the rust is keeping the vine in check by reducing its fitness and fecundity. Herb US holds undated records of *C. grandiflora*, based on images from Tsavo West National Park and the Mombasa Beach Hotel, where the species seems to be cultivated.

Namibia

Specimens at Herbs MO and US show records of *C. grandiflora* from the Namibian town of Karibib dating back to 1958. A Herb K record from the Etosha National Park in 2006 listed *C. grandiflora* as "fairly common"; whilst an earlier report from a game reserve bordering the Park described it as planted in "cultivated gardens", where it was linked to the poisoning and death of several elephants (Brain and Fox 1994).

South Africa

Henderson (2014) first highlighted the threat posed by *C. grandiflora* to grazing lands, riverine forests and woodlands in South Africa, subsequently detailing its escape from cultivation and the invasion of watercourses and pastoral land in the north-eastern Provinces of Limpopo and Mpumalanga (Sztab and Henderson 2015a). Equally, *C. madagascariensis* was listed as naturalised and potentially invasive (Sztab and

Henderson 2015b). Herb K records show the earliest collection of *C. grandiflora* from an arboretum in Pretoria in 1943; however, there are much earlier collections of *C. madagascariensis* from the botanic garden in Cape Town, dating from the 1860s and 1880s. Both *Cryptostegia* species are included on the 'Alien and Invasive Species List' of the National Environmental Management Biodiversity Act (NEMBA) and fall under environmental legislation for control (Government of South Africa 2020).

Tanzania

First collected in Tanga, north-east Tanzania, in 1929 (Herb EA), with early 1930s Herb K collections of *C. madagascariensis* (initially identified as *C. grandiflora*) from the nearby Moa District showing the annotation: "originally introduced as a rubber vine [presumably as a source of rubber] and now found in most gardens on the coast". However, there have been no reports of it as an invasive species (Witt et al. 2018). Similarly, *C. grandiflora* has also been recorded as an ornamental in Tanzania and is not listed as an invasive (Witt and Beale 2018).

Mascarenes

Mauritius

Bojer (1837) had listed *C. madagascariensis* as an exotic species in the flora of Mauritius and this is substantiated in an annotation by the botanist P. Koenig on a specimen in Herb K (originally from the Hooker Herbarium, deposited in 1867): "introduced here 2 or 3 centuries ago by the 'Malagasey' people who settled at the foot of the Signal Mountain, where it is most abundant". Whilst GISD and PIER (2022) list the species as native to Mauritius, referencing a 2013 version of GRIN, the latest version gives its status as naturalised (GRIN 2022). A record of *C. grandiflora* from the Hooker Herbarium in Herb K also documents this species as occurring in Mauritius. Neither species has been reported as invasive on the island.

Seychelles

Similar to Mauritius, GISD (2022) lists *C. madagascariensis* as native to the Seychelles, whilst other sources give its status as introduced and naturalised (Robertson and Todd 1983; GRIN 2022; POWO 2022). No further information is available for *C. grandiflora*, which is also recorded as being present.

Réunion

First introduced as an ornamental, *C. grandiflora* is now naturalised in the savannah areas. The species is listed as potentially invasive and as a threat to dry savannahs and pastures (Comité Français de L'UICN 2022; Groupe Espèces Invasives de La Réunion 2022).

North America

Mexico

Rodriguez-Estrella et al. (2010) indicated that *C. grandiflora* was introduced as an ornamental in the 1930s; however, a record in Herb US documents that the species has been present in Sinaloa, northwest Mexico, since the late 19th century. In 1924, it was reported to be naturalised in this Mexican state (Standley 1924) and recorded as invading dry rivers in Sonora in 1935, where it was said to be cultivated in gardens as an ornamental. In the early 1940s, *C. grandiflora* was also grown for research purposes as a source of rubber at the United States Cryptostegia Research Laboratory, Ciudad Victoria, Tamaulipas (Stewart et al. 1948). Present in at least 10 Mexican states (Patterson and Nesom 2009), investigations of the occurrence of *C. grandiflora* in natural oases in the dry region of the Baja California Peninsula found a high incidence, posing a significant threat to endemic species of oasis-dependent invertebrates and vertebrates (Rodriguez-Estrella et al. 2010). The species is now considered as invasive in Baja California as well as in Chiapas, Tabasco and Yucatan. Whilst *C. madagascariensis* is also known to be present in Baja California, Tabasco and Yucatan (Davidse et al. 2009), there is no reference to its being invasive.

USA

The earliest record for both *C. grandiflora* and *C. madagascariensis* is from the New York Botanical Garden in 1905. Both species are in cultivation in gardens and plant nurseries – especially in Florida, where they were introduced in the early 1900s (Polhamus et al. 1934) – and are usually marketed under the name purple allamanda. In Starr County, Texas, *C. grandiflora* has been reported to smother vegetation at sites along the Rio Grande (Patterson and Nesom 2009) and, based on climate matching, it has been classified as a high-risk invasive species in some southern states, notably Florida and Texas (Anon 2020). Considered by Meyer (2000) as a potential invader or a perceived threat to Hawaii, *C. grandiflora*, or more correctly, *C. madagascariensis* (fide Herb K), subsequently became invasive on several of the islands. The species was the subject of an apparently successful eradication campaign (Penniman et al. 2011), although this needs confirmation.

Central America and the Caribbean

Cryptostegia grandiflora and/or *C. madagascariensis* have been reported as present in all of the Central American countries, as well as on a number of the Caribbean islands; however, their respective status has been recorded as naturalised or invasive in less than half of the respective countries or territories (see Table 1).

Cuba

Records from the New York Botanical Garden (Herb NY) document that *C. grandiflora* was first collected in Cuba in the late 19th century; the species is now considered

as invasive (González-Torres et al. 2012). There is no information about the status of *C. madagascariensis*, first recorded on the island in 1926 (Herb US).

Haiti

The history of *Cryptostegia* in Haiti has been detailed above, as have the failed attempts to cultivate it as a source of rubber in the 1940s. Records in Herb US show that *C. madagascariensis* was already present in 1927, but there are no reports of the species becoming naturalised or weedy. *Cryptostegia grandiflora* is also listed as present (POWO 2022) and specimens dating from 1927 were deposited in Herb K and Herb NY. Nonetheless, there is uncertainty about their correct identification as they are duplicates of the earliest collection made from Haiti by Ekmann (GBIF 2021), which have been reliably identified as *C. madagascariensis*. No specimens of the high-yielding, *Cryptostegia* hybrids, vegetatively reproduced and planted for rubber production, were deposited in public herbaria. The fact that, following the collapse of the rubber project neither of these hybrids, nor their parental species, have become invasive could point to environmental constraints, as yet poorly understood.

Martinique

Cryptostegia madagascariensis, or allamanda pourpre, was introduced in the early 1900s as an ornamental: now naturalised in the dry forests in the south of the island where it is perceived as a potential invasive threat (Courty and Lasalle 2020). However, the rider is added that *C. grandiflora* is also 'appreciated' and cultivated as a climbing ornamental.

Montserrat

The earliest record of *C. madagascariensis* in Montserrat is unknown, but the species is now regarded as one of the key alien plants on the island and is being closely monitored. It has been described as "covering large tracts of land in the Silver Hills where it grows almost as a monoculture at the expense of other species" (Young 2008). First recorded in 1979 (Herb NY), *C. grandiflora* is considered as equally invasive (Varnham 2006).

Netherland Antilles

Buurt (2010) was precise about how *C. grandiflora* arrived in Curaçao; reporting that "the plant was imported from the area near Tulear in Madagascar during the First World War", as a potential source of rubber, although a record in Herb US indicates that the species was present on the island as early as March 1913. The author considered that, although the species was invasive on the island, it was debatable if it had a detrimental impact on the ecosystem. However, in the Christoffel National Park, in the north of the island, there is no doubt that *C. grandiflora* is having a negative impact, smothering native vegetation, especially members of the Cactaceae (Evans HC, pers.

obs. 2005, see Fig. 7). *Cryptostegia grandiflora* is also reported as invasive on Aruba and Bonaire, as naturalised on Saba and St Maarten/St Martin and as present on St Eustatius (Arnoldo 1971; Kairo et al. 2003; Burg et al. 2012). *Cryptostegia madagascariensis* is also recorded as present on Saba and St Eustatius (Herb NY; Mayfield-Meyer and Zhuang 2022), but there are no reports of this species being invasive.

St Lucia

Unknown when first introduced, *C. madagascariensis* is now considered to be naturalised on the island and is commonly found in the dry savannah, especially around Micoud, Vieux Fort and Laborie (Graveson 2021). The species is also cultivated as an ornamental. There is a record of *C. grandiflora* in Herb L from 1909, but further information about the invasive status of this species is lacking.

Virgin Islands

Following its introduction as an ornamental, *C. madagascariensis* has become invasive on the British Virgin Island of Anegada, where it is posing a threat to the island's biodiversity (McGowan et al. 2006). The species is also reported as naturalised on each of the three main U.S. Virgin Islands, St Croix, St John and St Thomas (Acevedo-

Figure 7. *Cryptostegia grandiflora* over-growing native vegetation in Christoffel National Park, Curaçao, Lesser Antilles, 2005.

Rodríguez 2005). *Cryptostegia grandiflora* has been recorded on the islands of Tortola (British Virgin Islands) and St Croix (U.S. Virgin Islands), without further details about its invasive status (GRIN 2022). Though previously also reported from St John (Acevedo-Rodríguez 1996), this identification was subsequently corrected by the author to *C. madagascariensis* (Acevedo-Rodríguez 2005).

South America

Brazil

Two herbarium specimens collected in 1906 around Manaus, Amazonas and deposited in Herb P, are probably the oldest records of *Cryptostegia* in Brazil, indicating the presence of rubber vine in this region during the peak of the first Amazon rubber boom. In 1916, Pickel reported the cultivation of *C. grandiflora* in Olinda, Pernambuco, on the north-east coast of Brazil on the label of a herbarium specimen kept at Herb IPA, which possibly constitutes the second oldest collection of the genus in Brazil. The first record of *C. madagascariensis*, also from Pernambuco, dates from 1930 (Herb US). However, there is anecdotal evidence that the species might have been present in the region much earlier in the form of a painting by José dos Reis Carvalhoos from 1859 depicting a reddish-flowering vine, resembling *Cryptostegia* sp., climbing up a carnaúba palm (Fig. 8).

The first indication that rubber vine was problematic in Brazil came in a report from the north-east region entitled 'dangerous visitors' (Herrera and Major 2006), highlighting the invasion of *C.* "*grandiflora*" ("cipó-de-sapo" or toad creeper). The species was invading riverine forests and posing a threat to the forests of native carnaúba palm (*Copernicia prunifera*, Arecaceae). Subsequently, two of the present authors (Barreto

Figure 8. Watercolour painting "Corte de carnauba" by José dos Reis Carvalhoos (1859) depicting a red-purple flowering vine, potentially *Cryptostegia* sp., climbing up a carnaúba palm (right-hand side) (source: Wikimedia Commons, public domain).

RW and Evans HC) visited the region and confirmed these findings – in particular, its impact on the ecologically and economically important carnaúba or wax palm (Fig. 9A). However, the rubber vine species involved turned out to be *C. madagascariensis* (da Silva et al. 2008) and the plant was more commonly known as 'unha-do diabo' or devil's claw, due to the claw-like appearance of the fruits (Fig. 9B). It was posited that this material may have originated from the rubber-vine collection in the Rio de Janeiro Botanical Garden, which was misidentified as *C. grandiflora*, with the ubiquitous common name purple allamanda ('alamanda-roxa'; Fig. 9C). Molecular evidence corroborates this supposition (Authors, unpubl. data), although the oldest collections of *Cryptostegia* in the south-east region date from the 1940s, decades after the first records in the north and north-east of the country. While *C. grandiflora* has also been reported from several Brazilian states, listed as naturalised, but not yet invasive (Table 1), it is possible that these records are based on a misidentification of *C. madagascariensis*.

Studies show that *C. madagascariensis* is having a significant negative impact on the unique semi-arid Caatinga ecosystem in north-east Brazil, affecting the regeneration and ecological succession of native vegetation (Sousa et al. 2016), as well as altering the composition of arbuscular mycorrhizal communities (Souza et al. 2016). However, it is the socio-economic impact on the carnaúba palm that is the main cause of concern in the region since, as well being an emblematic and keystone species in the States of Ceará, Piauí and Rio Grande do Norte, *C. prunifera* is an important source of income and rural employment. In 2019, export of the high-quality wax obtained from the palm leaves was valued at over US$ 40 million to the Brazilian economy (IBGE 2019). This has been the main driver behind a collaborative project funded by private industry and the government of Ceará to assess the potential of the rust fungus *M. cryptostegiae* from Madagascar as a CBC agent, in an attempt to replicate the success of this strategy in managing the congeneric species *C. grandiflora* in Australia (Evans 2000, 2013). Within the scope of this Brazilian project, which commenced in 2018, surveys for fungal pathogens in the native Madagascan range of *C. madagascariensis* and subsequent screening in the UK under quarantine greenhouse conditions, identified a strain or pathotype of *M. cryptostegiae* highly virulent to the only known invasive biotype of *C. madagascariensis*. Host-specificity testing of the selected pathotype against 48 non-target plants representative of native Brazilian apocynaceous genera and species, as well as locally-important species from other plant genera, showed it to be specific to the genus *Cryptostegia*. In parallel, field studies were conducted in Ceará to collate data on *C. madagascariensis* populations and plant performance in order to establish a baseline against which future impacts of the rust can be assessed. If approved for release, *M. cryptostegiae* would be the first exotic weed biocontrol agent introduced into Brazil.

Colombia

Gracia et al. (2019) considered that *C. madagascariensis* was originally introduced into the resorts on the Caribbean coast of Colombia as an ornamental and described how it is now forming impenetrable thickets, covering trees and displacing indigenous dune

Figure 9. *Cryptostegia madagascariensis* **A** completely smothering native riparian vegetation and climbing up the endemic palm, *Copernicia prunifera* (Carnaúba or wax palm), Cruz, Acaraú River, Ceará, Brazil, 2007 **B** close-up of flowers and fruits; showing their claw-like nature, particularly of the opened fruits **C** in the Botanic Garden, Rio de Janeiro, with erroneous identification (inset), showing the characteristic whip-like shoots.

vegetation. Nonetheless, a record of *C. madagascariensis* in Herb US dates as early as 1899. The same herbarium also holds a record of *C. grandiflora* showing its presence in Colombia in 1906, while out of two authenticated *C. grandiflora* records in Herb K (Klackenberg 2001), the earlier one from Guajira near the Caribbean coast dates from

1917. It is likely, therefore, that multiple introductions of *Cryptostegia* species have taken place with the reported invasion of the dune ecosystem resulting from a more recent introduction event.

Ecuador

Gardener et al. (2010) discussed an eradication programme on the Galápagos Islands in which *C. grandiflora* was included, based on its past history as an invasive species elsewhere rather than on its spread within the islands. According to Guézou et al. (2010), the plant was detected only in gardens on Santa Cruz Island. Later, Buddenhagen and Tye (2015) discussed the programme and concluded that *C. grandiflora* had been "almost eradicated by 2007", although they noted that its management had since been abandoned. There is also a record of this species on mainland Ecuador from 1926 (Herb US), but there is no further information of its current presence or weed status.

Oceania

Australia

Tomley (1995) accessed published records from several botanical gardens in Brisbane and concluded that *C. grandiflora* was introduced into Australia as an ornamental in the late 19th century. It seems credible that it was sent from a botanical garden in India rather than arriving directly from Madagascar, although there is no evidence to support this supposition. Rubber-vine weed, as it became known, was reported as being weedy in Queensland some decades later, but it only became a problematic invasive weed following attempts to establish it as a source of rubber during the Second World War in the mining areas of central Queensland (Tomley 1995). By 1990, *C. grandiflora* was estimated to cover over 30,000 km^2 in tropical Queensland and was described as "the single greatest threat to biodiversity in tropical Australia" (Fig. 10A) (McFadyen and Harvey 1990). Based on climatic suitability, it was calculated

Figure 10. A *Cryptostegia grandiflora* climbing up into the canopy and smothering native *Eucalyptus* stands, northern Queensland, Australia **B** *C. grandiflora* thicket showing immediate impact of the rust, *Maravalia cryptostegiae*, with yellowing and falling leaves, three months after its release.

that the species had the potential to invade up to 160,000 km^2 (Tomley 1995), which has since been supported by CLIMEX modelling. This would put the whole of the Northern Territory and northern Western Australia at risk of invasion with severe implications for natural ecosystems, including World Heritage areas, such as Kakadu National Park (Kriticos et al. 2003).

This actual and potential threat to the ecosystems of tropical Australia was the catalyst for an integrated management strategy – including a CBC programme – implemented by the then Queensland Department of Lands. This was funded in part by the Australian Meat and Livestock Research and Development Corporation because of the impact of rubber-vine weed on the cattle industry due to loss of grazing and reduced access to water sources by weed infestations (McFadyen and Harvey 1990; Tomley 1995). Surveys in Madagascar for natural enemies of *Cryptostegia* identified several promising CBC agents; including the aforementioned damaging rust fungus, *M. cryptostegiae*. This rust has since been recognised as closely related to the genera *Elateraecium* and *Hemileia*, both phylogenetically distant from *Maravalia* and has temporarily been placed in the genus *Uredo* (Aime and McTaggart 2020). Thus, a new generic name will be needed to accommodate the rust species on *Cryptostegia* from Madagascar. It is also considered that this rust genus will prove to be unique to Madagascar; having co-evolved with its endemic plant host.

Following extensive safety testing, the Madagascan moth, *Euclasta whalleyi* (Pyralidae, Lepidoptera), as well as the rust fungus, were released in northern Australia in the late 1980s and early 1990s. Long-term monitoring studies of the rust have shown significant impacts on weed populations with much-reduced seedling recruitment (Fig. 10B) (Vogler and Lindsay 2002; Tomley and Evans 2004). An economic impact assessment put the net benefit of the project at over AU$ 230 million, with a benefit-cost ratio of 108:1 (Page and Lacey 2006), making it one of the most successful weed biocontrol programmes in Australia (Palmer et al. 2010; Evans 2013).

Cryptostegia madagascariensis is present in the Northern Territory, Queensland and Western Australia (Marohasy and Forster 1991; Atlas of Living Australia 2023a) but, to date, has not been reported as invasive in these States (Taylor D, pers. com. 2022).

Oceanian Islands

Cryptostegia grandiflora has been cultivated on a number of the islands. For Papua New Guinea, there is a Herb K record dated 1936 from the New Guinea Agricultural Department, labelled *Cryptostegia* sp., with the annotation: "from which fibre is prepared". Presumably, therefore, it was being grown as a crop for rope or similar products. While classed as established on several of the islands (see Table 1), *C. grandiflora* is described as a moderate invader only in New Caledonia (Meyer 2000). However, the same author considers the species also as a potential invader on Fiji. *Cryptostegia madagascariensis* has been reported only from the Cook Islands, French Polynesia and Palau – being less widely distributed in the region – and there are no reports of the species as an invasive.

Conclusions

The two representative species of *Cryptostegia* native to Madagascar are now present in most countries of the sub-tropics and tropics, including remote island systems. These species – commonly and collectively known as rubber vines – were introduced initially for their ornamental value, but later, prior to and during both World Wars, they were also cultivated as potential sources of rubber. In many, but not all countries, these two vines have become naturalised and, in several, they have assumed the status of an invasive weed posing a threat to indigenous ecosystems, as well as to agriculture. From the data available, the two rubber vines appear to be 'sleeper weeds' in the sense that many years may elapse from their escape and naturalisation to becoming invasive and problematic (Groves 2006). For example, *C. madagascariensis* has been cultivated in the north-east of Brazil since 1916, but it was a further 90 years before it was reported as invasive in this region. The reasons are unclear, but abiotic factors, such as soil type, climate change and/or habitat disturbance, may be involved. The weed status of *C. grandiflora* in India appears to be more complicated and difficult to interpret as there is no evidence to suggest that this species – several centuries since the first confirmed report of its presence – has become invasive or problematic, despite Kriticos et al. (2003) identifying extensive areas in southern India as being highly suitable for its growth, based on climatic data.

The Australian experience shows that CBC can be successful in controlling rubber vine invasions, provided the invasive *Cryptostegia* species and biotype is correctly matched with a respective pathotype of the rust *M. cryptostegiae*. Hopefully, this success can be replicated in Brazil and, potentially, other countries affected by invasive rubber vines should they embrace this control approach in the future. Nonetheless, the message would appear to be that, despite its attraction as an ornamental and perceived usefulness as a source of rubber, caution should be exercised concerning their potential to become invasive wherever the two species have been introduced, as well as posing a threat to human health, in addition to that of livestock and herbivores, in general, due to toxic glycosides in the latex (McFadyen and Harvey 1990; Brain and Fox 1994; Albuquerque et al. 2009; Alasbahi and Al-Hawshabi 2021). Their cultivation as ornamentals in public and private gardens must be discouraged and their commercialisation should be forbidden by law. At present, even in some places severely impacted by rubber vines, such as in north-east Brazil, it is still being deliberately cultivated which is likely to be contributing directly to the expansion of its distribution and the resulting negative impacts.

Author contributions

Marion Seier and Harry Evans conceptualised and put together the initial drafts of the manuscript. Alessandro Rapini, Marion Seier and Kate Pollard collated, validated and curated species distribution data. All authors reviewed, edited and approved the final manuscript.

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RESEARCH ARTICLE

Genetic and morphological insights into the *Carpobrotus* hybrid complex around the world

Ana Novoa[1], Heidi Hirsch[2], María L. Castillo[1], Susan Canavan[1,3],
Luís González[4], David M. Richardson[1,5], Petr Pyšek[1,6],
Jonatan Rodríguez[1,7], Lurdes Borges Silva[8,9,10], Giuseppe Brundu[11],
Carla M. D'Antonio[12], Jorge L. Gutiérrez[13,14], Megan Mathese[15],
Sam Levin[16,17], Luís Silva[8,9,10,18], Johannes J. Le Roux[5,19]

1 *Department of Invasion Ecology, Institute of Botany, Czech Academy of Sciences, 25243 Průhonice, Czech Republic* **2** *Lynedoch Road, Stellenbosch 7600 South Africa* **3** *School of Natural Sciences, Ollscoil na Gaillimhe, University of Galway, Galway, Ireland* **4** *Plant Ecophysiology Group: Invasive plants, Department of Plant Biology and Soil Sciences, Universidade de Vigo, 36310, Vigo, Spain* **5** *Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa* **6** *Department of Ecology, Faculty of Science, Charles University, Viničná 7, 12844 Prague, Czech Republic* **7** *CRETUS, Department of Functional Biology, Faculty of Biology, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain* **8** *CIBIO, Research Center in Biodiversity and Genetic Resources, InBIO Associate Laboratory, Pólo dos Açores, Universidade dos Açores, Campus de Ponta Delgada, Rua da Mãe de Deus, 9500-321 Ponta Delgada, Açores, Portugal* **9** *BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal* **10** *UNESCO Chair – Land Within Sea: Biodiversity & Sustainability in Atlantic Islands, Universidade dos Açores, Campus de Ponta Delgada, Rua da Mãe de Deus, 9500-321 Ponta Delgada, Açores, Portugal* **11** *Department of Agricultural Sciences, University of Sassari, 07100 Sassari, Italy* **12** *Environmental Studies Program, University of California, Santa Barbara 93106, USA* **13** *Facultad de Ciencias Exactas y Naturales, Instituto de Geología de Costas y del Cuaternario (IGCyC, UNMdP-CIC) and Instituto de Investigaciones Marinas y Costeras (IIMyC, UNMdP-CONICET), Universidad Nacional de Mar del Plata, Mar del Plata, Argentina* **14** *Grupo de Investigación y Educación en Temas Ambientales (GrIETA), Estación Biológica Las Brusquitas, San Eduardo del Mar, Argentina* **15** *Department of Botany & Zoology, Stellenbosch University, Matieland 7602, South Africa* **16** *Martin Luther University Halle-Wittenberg, Institute of Geobotany, Am Kirchtor 1, 06108 Halle (Saale), Germany* **17** *German Centre for Integrative Biodiversity (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany* **18** *Faculty of Sciences and Technology, University of the Azores, Rua da Mãe de Deus, 9500-321 Ponta Delgada, Portugal* **19** *School of Natural Sciences, Macquarie University, Sydney, NSW 2109, Australia*

Corresponding author: Ana Novoa (novoa.perez.ana@gmail.com)

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Abstract

The genus *Carpobrotus* N.E.Br. comprises between 12 and 25 species, most of which are native to South Africa. Some *Carpobrotus* species are considered among the most damaging invasive species in coastal dune systems worldwide. In their introduced areas, these species represent a serious threat to native species and significantly impact soil conditions and geochemical processes. Despite being well studied, the taxonomy of *Carpobrotus* remains problematic, as the genus comprises a complex of species that hybridize easily and are difficult to distinguish from each other. To explore the population genetic structure of invasive *Carpobrotus* species (i.e., *C. acinaciformis* and *C. edulis*) across a significant part of their native and non-native ranges, we sampled 40 populations across Argentina, Italy, New Zealand, Portugal, South Africa, Spain, and the USA. We developed taxon-specific microsatellite markers using a Next Generation Sequencing approach to analyze the population genetic structure and incidence of hybridization in native and non-native regions. We identified three genetically distinct clusters, which are present in both the native and non-native regions. Based on a set of selected morphological characteristics, we found no clear features to identify taxa morphologically. Our results suggest that the most probable sources of global introductions of *Carpobrotus* species are the Western Cape region of South Africa and the coastline of California. We suggest that management actions targeting *Carpobrotus* invasions globally should focus on preventing additional introductions from the east coast of South Africa, and on searching for prospective biocontrol agents in the Western Cape region of South Africa.

Keywords

Biological invasions, genetic diversity, genetic structure, hybridization, introduction history, invasive alien plant, microsatellite markers, taxonomic uncertainty

Introduction

Coastal habitats such as coastal dunes, sea cliffs, and coastal prairies are exposed to a variety of extreme environmental conditions, including high salinity, low soil moisture, soil nutrient deficiencies, and intense wind and solar irradiance (Maun 2009). These conditions result in a high degree of specialization among species that naturally occur in these habitats (Mayoral et al. 2021). As such, coastal areas often host rare and endemic communities of high conservation value (Acosta et al. 2009). But coastal areas are also among the most endangered habitats (Defeo et al. 2009) and several anthropogenic drivers threaten their conservation, including biological invasions, climate change, habitat degradation, and urbanization (Carboni et al. 2009; Dawson et al. 2017). Invasive plants are considered to be one of the main threats to the conservation of the biodiversity and ecosystem functioning of coastal areas across the world (Millennium Ecosystem Assessment 2005).

The succulent genus *Carpobrotus* N.E.Br. (family Aizoaceae) comprises between 12 and 25 species and lower-rank taxa, most of them native to South Africa (Hartmann 2002). Several of these species are considered to be among the most widespread and damaging invasive plants in coastal areas globally (Campoy et al. 2018). *Carpobrotus* taxa have been introduced to coastal areas accross the world for ornamental purposes and for soil and dune stabilization. For example, they have been present in European gardens

since the late 17th century (Preston and Sell 1988) and, in California, they have been used for soil stabilization since the early 20th century (Albert et al. 1997). *Carpobrotus* species have invaded millions of hectares of coastal areas worldwide, including in Argentina, Australia, California, Chile, New Zealand, and Southern and Western Europe (Campoy et al. 2018), impacting biodiversity and native species community structure and ecosystem functioning in multiple ways. For example, they compete with native plants for space, nutrients and water, reducing their growth, survival, and reproduction (D'Antonio and Mahall 1991; Molinari et al. 2007; Novoa and González 2014). They are also considered ecosystem engineers (Cuddington et al. 2011) since they can cause substantial and irreversible changes to invaded soils (Novoa et al. 2014). In particular, dense patches of invasive *Carpobrotus* produce and accumulate large amounts of litter (Fenollosa et al. 2016), which increases soil water holding capacity and, during its decomposition, decreases soil pH, and increases soil nutrient contents (Novoa et al. 2012, 2014). These changes 'soften' the extreme environmental conditions typical of coastal areas and facilitate the establishment and growth of opportunistic weeds while replacing native coastal vegetation (Novoa et al. 2012, 2013). Invasive *Carpobrotus* also alters the diversity, composition and functioning of soil microbial (Lechuga-Lago et al. 2017; Novoa et al. 2020) and invertebrate communities (Rodríguez et al. 2020; Gutiérrez 2021) and disrupts native pollination (Jakobsson et al. 2008) and herbivory networks (Rodríguez et al. 2019, 2021).

To gain insight into the invasiveness and impact of non-native species, as well as to develop or improve management actions it is important to know the taxonomic identity and the introduction history of the target invasive species (Pyšek et al. 2013). However, the taxonomy and biogeography of *Carpobrotus* spp. have long been a subject of debate (Campoy et al. 2018). Most of the taxa are native to South Africa, but five are native to Australia, and one species (*C. chilensis*) may be native to the Americas. *Carpobrotus* spp. have been described in several floras worldwide (Harvey and Sonder 1861; Blake 1969; Bolus Herbarium Collection 2015; Preston and Sell 1988; Gonçalves 1991; Wisura and Glen 1993), but these lists do not use the same traits to delineate species. The main diagnostic characters used to differentiate species are flower color and shape of the leaf section. However, there are doubts over the validity of these traits for identifying *Carpobrotus* species (Campoy et al. 2018). Thus, the information given in these documents cannot be easily synchronized or compared (Hartmann 2002). Moreover, due to their succulence, *Carpobrotus* spp. are difficult to curate, and therefore are poorly represented in herbarium collections (Walters et al. 2011). In fact, in several cases, the species names are based on lectotypes selected from illustrations, e.g., by Dillenius (1732). As a result, the taxonomy of the genus remains problematic.

Two *Carpobrotus* species are currently considered to be invasive: *C. edulis* (L.) N.E.Br., and *C. acinaciformis* (L.) L.Bolus (Campoy et al. 2018). *Carpobrotus edulis* is the most popular and widely introduced species in the genus. It is native to South Africa and considered one of the worst invasive plants of coastal areas and one of the most thoroughly studied invasive species worldwide (Pyšek et al. 2008; Campoy et al. 2018). It has been reported to hybridize with other *Carpobrotus* species in its native and invasive ranges (hybrids have been documented in the Americas, Australia, Europe, and South

Africa; e.g., Campoy et al. 2018). Hybrids between *C. edulis* and species from other genera (e.g., *Sarcozona*) have also been reported outside South Africa (e.g., Heenan and Sykes 2010). *Carpobrotus acinaciformis* is generally considered to be native to South Africa, although it has also been suggested that it may be a hybrid between *C. edulis* and other South African or Australian congeners (Schierenbeck et al. 2005). *Carpobrotus edulis* and *C. acinaciformis* have a long history of human use in South Africa, and therefore, their natural limits and identities may also be conflated (Malan and Notten 2006).

  Carpobrotus chilensis also provides a good example of the taxonomic and biogeographic uncertainties that plague the genus. Some authors consider this species to be native to California and Chile (Brown 1928), while others regard it as native to Argentina and Chile (Hartmann 2002; Zuloaga and Belgrano 2017; US National Plant Germplasm System 2022) and still others suggest it is "probably native to South Africa" (https://ucjeps. berkeley.edu/eflora/eflora_display.php?tid=77164). An extensive review of herbarium and historical records carried out to identify the origin of this species was inconclusive (Bicknell and Mackey 1988). Some authors have even considered it to be a hybrid swarm of five South African species (i.e., *C. deliciosus*, *C. dimidiatus*, *C. edulis*, *C. mellei*, and *C. muirii*) (Bicknell and Mackey 1998). Hence, the origins and taxonomic classification of this taxon are speculative at best. In California, *C. chilensis* has been reported to hybridize with the South African *C. edulis* (Gallagher et al. 1997; Albert et al. 1997; Vilà and D'Antonio 1998) with extensive directional backcrossing and potential loss of pure *C. chilensis* types (Vilà et al. 1998; Schierenbeck et al. 2005). Overall, the genus *Carpobrotus* is often considered to be a complex of species that easily hybridizes and are difficult to distinguish (Traveset et al. 2008). This taxonomic uncertainty is further complicated by the clonal growth typical of the genus, which stabilizes hybrid genotypes (Ellstrand and Schierenbeck 2000).

  Here, we aim to shed light on the relatedness and introduction history of invasive *Carpobrotus* spp. around the world. With this overarching aim, we (1) sampled invasive *Carpobrotus* species in coastal areas across many of their presumed native and invaded ranges and (2) developed and used a set of genus-specific microsatellite markers to assess and compare the genetic diversity and structure among these populations. Moreover, aiming to help managers and other stakeholders with the identification of invasive *Carpobrotus* species in the field, we (3) compared the morphological characteristics of the *Carpobrotus* taxa assigned to distinct genetic clusters.

Methods

Study areas and sampling

We sampled a total of 40 *Carpobrotus* populations distributed across their native and invasive ranges (Fig. 1). We considered coastal areas up to 1 km from the sea since these are the typical habitats of invasive *Carpobrotus* taxa (i.e., *C. acinaciformis* and *C. edulis*) (Campoy et al. 2018). According to Hartmann (2002), in South Africa there are six *Carpobrotus* species growing in proximity to the coast: *C. acinaciformis*, *C. muirii* and *C. quadrifidus* found in the Western Cape province; *C. edulis* distributed through
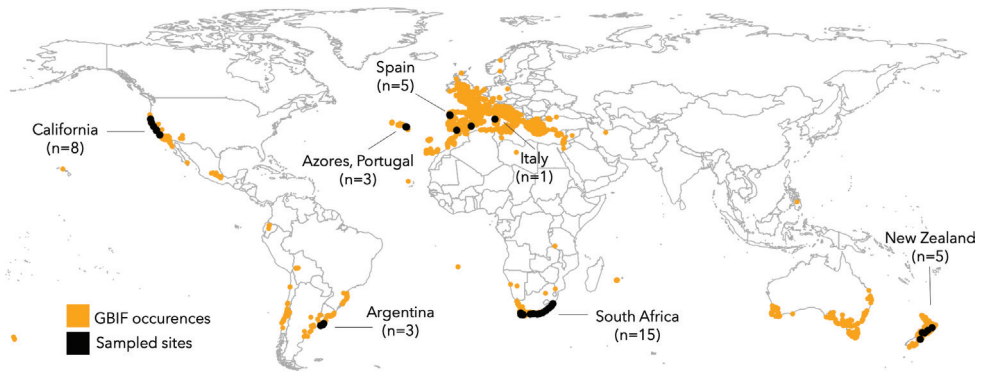
Figure 1. Populations of *Carpobrotus* species sampled in this study (see Table 1 for further details). Orange points indicate occurrence records of invasive *Carpobrotus* species (i.e., *C. acinaciformis* and *C. edulis*) extracted from the GBIF database (gbif.org; downloaded: 31 March 2023; https://doi.org/10.15468/dl.j637g9). Black points indicate the locations where *Carpobrotus* populations were sampled in this study. Numbers in brackets indicate the number of populations sampled per region.

the Northern Cape province, Western Cape and Eastern Cape provinces; *C. deliciosus*, which occurs in the Western Cape, Eastern Cape and KwaZulu-Natal provinces; and *C. dimidiatus*, found in the Eastern Cape and KwaZulu Natal provinces (Smith et al. 1998). Therefore, we selected 15 *Carpobrotus* populations distributed along most of South Africa's coastline (Fig. 1). The selection of the remaining populations was based on the current distribution of invasive *Carpobrotus* species across the world. We extracted information on the current spatial distribution of *C. acinaciformis* and *C. edulis* from the Global Biodiversity Information Facility (GBIF, gbif.org; downloaded: 31 March 2023; https://doi.org/10.15468/ dl.j637g9). We kept records categorized as human observations, literature, living specimens or observations (Fig. 1). Based on these records, we selected 25 additional populations across the observed ranges (Fig. 1, Table 1).

We excluded Chile from our studied area due to issues encountered with exporting plant material from that country. *Carpobrotus* species are also found all along Australia's coastline (Fig. 1). According to Hartmann (2002), four species (i.e., *C. glaucescens*, *C. modestus*, *C. rossii* and *C. virescens*) are native to the country. Moreover, *Carpobrotus* spp. in Australia are hybridizing, both among species within the genus and with species in the genus *Sarcozona* (Campoy et al. 2018). Due to this, and the fact that none of the *Carpobrotus* species native to Australia are recorded as invasive elsewhere in the world, and they are clearly distinct from *C. chilensis* (Bicknell and Mackey 1998), we decided to exclude Australia from our sampling effort.

In each locality (Fig. 1, Table 1), we sampled fresh leaves from about 20 randomly chosen ramets per population (total $n = 20$ ramets $\times$ 40 populations = 800 ramets). The minimum distance between sampled ramets in each population was 5 m. The fresh collected material was shipped to Stellenbosch University, South Africa. Local regulations for sample collection and shipment were followed. Because some samples failed for our molecular analyses, sample sizes for some populations are <20 (Table 1).

Table 1. Locality details and genetic characteristics of populations of *Carpobrotus* species sampled in this study (also see Fig. 1). The region, locality, latitude (Lat) and longitude (Long) in decimal degrees (WGS84), number of samples used for genotyping (N), assigned genetic cluster (Cluster; also see Fig. 4), mean values for the number of alleles per locus (Na), observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}) are provided for each sampled population. For clonal diversity, the number of genotypes (G), number of effective alleles (Ne), Nei's (1987) genetic diversity corrected per sample size (H_e) and Nei's uncorrected genetic diversity (Gd) are presented. *diversity-based test not significant, indicating that clonal copies are not necessarily the result of asexual reproduction. The test was significant for all remaining populations (See Methods section for more details). Samples from Cape Point (ZA8) had excessive missing genotype data and were thus removed from the analyses.

ID	Region	Locality	Coordinates (Lat, Long)	N	Cluster	Genetic diversity				Clonal diversity			
						Na	H_O	H_E	F_{IS}	G	Ne	H_e	Gd
NZ1	New Zealand	Whirinaki	-39.829, 176.8914	10	A	2.167	0.483	0.335	-0.440	8	6.250	0.933	0.840
NZ2	New Zealand	Foxton	-40.4557, 175.2168	10	A	2.167	0.533	0.336	-0.490	7	6.250	0.933	0.840
NZ3	New Zealand	Rough Island	-41.2709, 173.1137	14	A	1.667	0.524	0.299	-0.707	4	2.882	0.703	0.653
NZ4	New Zealand	Rarangi	-41.4188, 174.0357	20	A	1.833	0.550	0.336	-0.634	8	4.255	0.805	0.765
NZ5	New Zealand	Lake Ellsmere	-43.8599, 172.3534	20	A	2.000	0.542	0.367	-0.393	11	9.524	0.942	0.895
SE1	Azores	São Vicente	37.8325, -25.6647	30	A	1.500	0.417	0.229	-0.778	1	1.000	0	0
SE2	Spain	Punta de Rons	42.497, -8.8790	16	A	1.500	0.500	0.250	-1.000	1	1.000	0	0
SE3	Spain	A Lanzada	42.4328, -8.875215	24	A	1.500	0.500	0.250	-1.000	1	1.000	0	0
ZA1	South Africa	Rooisand	-34.3490, 19.0909	16	A	2.500	0.469	0.330	-0.344	7	2.415	0.625	0.586
ZA3	South Africa	Vogelgat	-34.4021, 19.3199	16	A	2.500	0.533	0.358	-0.308	7	3.879	0.792	0.742
ZA4	South Africa	Belvidere	-34.0532, 22.9964	13	A	2.000	0.474	0.368	-0.201	8	6.259	0.910	0.840
CA2	California	Celeste	40.8520, -124.1710	23	B	1.333	0.341	0.174	-0.674	1	1.000	0	0
CA3	California	Point Reyes	38.0457, -122.9888	20	B	2.167	0.544	0.431	-0.279	11	8.333	0.926	0.880
CA4	California	For Ord	36.6587, -121.8226	20	B	2.333	0.563	0.427	-0.320	15	10.526	0.953	0.905
CA5	California	Soberanes Point	36.45065, -121.9280	19	B	1.833	0.536	0.358	-0.399	4	2.391	0.614	0.582
CA6	California	Minuteman beach	34.8563, -120.6086	8	B	1.833	0.542	0.296	-0.736	3	2.133	0.607	0.531
CA7	California	Wall beach	34.70521, -120.5995	18	B	2.333	0.576	0.418	-0.341	10	7.364	0.915	0.864
CA8	California	South Base	34.70520, -120.6012	7	B	2.333	0.494	0.422	-0.179	5	3.769	0.857	0.735
SE6	Azores	Ribeira Grande	37.8305, -25.5163	28	B	1.667	0.648	0.333	-0.947	1	1.000	0	0
SE7	Spain	Samil	42.2144, -8.7755	20	B	1.500	0.500	0.250	-1.000	1	1.000	0	0
SE8	Spain	Marina	38.1443, -0.6343	20	B	1.833	0.333	0.212	-0.232	2	1.220	0.189	0.180
ZA5	South Africa	Mdumbi	-31.9443, 29.2100	15	B	1.333	0.333	0.167	-1.000	1	1.000	0	0
ZA10	South Africa	Cape St Francis	-34.1766, 24.8231	8	C	1.667	0.229	0.142	-0.300	4	2.286	0.643	0.562
ZA11	South Africa	Port Elizabeth	-34.0247, 25.6480	19	C	2.167	0.364	0.251	-0.322	7	4.056	0.795*	0.753
ZA12	South Africa	Port Alfred	-33.6093, 26.8900	19	C	1.333	0.225	0.131	-0.606	2	1.870	0.491*	0.465
ZA13	South Africa	Cintsa	-32.8268, 28.1194	19	C	2.000	0.322	0.258	-0.232	3	1.994	0.526	0.499
ZA14	South Africa	Port Edward	-31.0441, 30.2276	18	C	1.500	0.250	0.166	-0.502	4	2.945	0.699	0.660
ZA9	South Africa	Keurboomstrand	-34.0286, 23.3975	20	C	2.000	0.400	0.270	-0.434	8	5.405	0.858	0.815
ARG1	Argentina	Mar Chiquita	-37.7550, -57.4304	22	Admixed	2.000	0.424	0.309	-0.251	4	1.967	0.515	0.492
ARG2	Argentina	San Eduardo del Mar	-38.2355, -57.7548	10	Admixed	2.000	0.412	0.339	-0.254	6	4.167	0.844	0.760
ARG3	Argentina	Quequén	-38.5675, -58.6499	9	Admixed	1.500	0.500	0.250	-1.000	1	1	0	0
CA1	California	Mackerricher	39.4912, -123.7950	16	Admixed	1.500	0.500	0.250	-1.000	3	2.415	0.625	0.586
SE5	Azores	Mosteiros	37.8986, -25.8175	36	Admixed	1.500	0.343	0.184	-0.507	2	1.117	0.108	0.105
SE4	Spain	Cádiz	36.5678, -6.2225	12	Admixed	1.833	0.475	0.315	0.330	4	2.880	0.712	0.653
SE9	Italy	Marina di Sorso	40.8194, 8.4953	21	Admixed	1.833	0.443	0.276	-0.484	6	2.96	0.695	0.662
ZA2	South Africa	Springfontein	-34.4287, 19.4065	10	Admixed	2.333	0.494	0.381	-0.362	10	10	1*	0.900
ZA6	South Africa	Mossel Bay	-34.1715, 22.1226	20	Admixed	2.667	0.507	0.444	-0.171	14	10.526	0.953	0.905
ZA7	South Africa	Melkbosstrand	-33.7065, 18.4482	17	Admixed	2.333	0.331	0.303	-0.048	4	2.513	0.64	0.602
ZA8	South Africa	Cape Point	-34.3530, 18.4888	17	–	–	–	–	–	–	–	–	–
ZA15	South Africa	Durban	-30.1268, 30.8457	18	Admixed	2.500	0.400	0.370	-0.170	8	3.951	0.791	0.747

Microsatellite development and genotyping

Microsatellite sequences were isolated by Ecogenics GmbH (Balgach, Switzerland). Size selected fragments from *Carpobrotus* genomic DNA were enriched for microsatellite repeats by using magnetic streptavidin beads and biotin-labelled CT and GT repeat oligonucleotides. The microsatellite enriched library was analyzed on a Roche 454 Titanium technology (Roche Diagnostics Corporation). This resulted in 89 reads containing microsatellite motifs of at least six microsatellite nucleotide repeat units. Suitable primer design was possible for 32 reads, of which 25 primer pairs were selected and tested for amplification and polymorphism. We extracted DNA from *Carpobrotus* leaf material using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle 1991). To assess initial amplification success and polymorphism, 25 selected simple sequence repeats (SSRs) loci were first amplified in ten *Carpobrotus* ramets collected in South Africa, using unlabelled primers. Each 10 μL reaction contained 2 μL genomic DNA (100 ng/μl), 1 uL 10× buffer, 200 mM dNTPs, 5 μM of each primer, 1 unit of Taq polymerase (Super-Therm JMR-801, Separations Scientific, Cape Town, South Africa), 0.2 μL bovine serum albumin (BSA, 10 mg/ml) and 3.6 μL of distilled water. The PCR cycling was as follows: 95 °C for 5 min, 30 cycles of 60 s at 94 °C, 60 s at primer-specific annealing temperature, 2 min at 72 °C, and a final elongation of 10 min at 72 °C. To detect polymorphism, the resulting PCR products were purified and run on an Agilent 2100 Bioanalyser analysis LabChip (Agilent Technologies). Out of the 25 loci tested, 19 were monomorphic across all the tested *Carpobrotus* individuals. The forward primers of the remaining six loci were fluorescently labelled with either HEX, 5-FAM, PET, or NED. Primer pairs were combined into two separate multiplex reactions and amplified in all *Carpobrotus* specimens. Each 15 μL multiplex reaction contained 3 μL genomic DNA (20 ng/μL), 1.5 uL primer mix (2 μM), 7.5 μL Qiagen multiplex PCR mix, and 3 μL Q-solution. PCR conditions for all multiplexes consisted of denaturation at 95 °C for 15 min, followed by 30 cycles of 30 s at 94 °C, 90 s at 55 °C, 60 s at 72 °C, and a final elongation of 30 min at 60 °C. Labelled PCR products were sent to the Central Analytical Facility, Stellenbosch University, Stellenbosch, South Africa, for fragment analysis. LIZ500 was used as the internal size standard. GeneMarker software (version 2.6.4; SoftGenetics LLC, Pennsylvania, USA) was used for genotype scoring by using marker panels to call the alleles. All allele scores were checked manually.

Dataset characteristics and genetic diversity

We used the software Micro-Checker (version 2.2; Van Oosterhout et al. 2004) to check our genotype dataset for the presence of scoring errors and null alleles. This software calculates expected homozygote and heterozygote allele size differences by assuming Hardy-Weinberg equilibrium (HWE) conditions, generating the frequency of expected and detected null alleles and applying a Monte Carlo simulation method (Van Oosterhout et al. 2004). Null alleles are identified at a given locus when HWE conditions among genotypes are rejected and if excess homozygous genotypes are evenly distributed among allele size classes. The presence of null alleles can bias calculations of F_{ST} values

and may lead to overestimation of population differentiation (Kim and Sappington 2013). Therefore, for more detailed estimates of null allele frequencies at each locus and population, the expected maximization method as implemented in the software FreeNA (Chapuis and Estoup 2007) was applied. FreeNA was also used to calculate uncorrected and corrected (i.e., excluding null alleles; so-called ENA method as described in Chapuis and Estoup 2007) pairwise F_{ST} values (Weir 1996). For all loci, allele frequency departures from HWE expectations were tested using the packages "adegenet"(version 2.1.1; Jombart 2008) and "pegas" (version 0.11; Paradis 2010) in R (version 3.5.3).

Linkage disequilibrium was evaluated with the "poppr" package (version 2.9.3; Kamvar et al. 2014) by using the index of association of alleles at different loci. For this, measures of correlation ($\bar{r}_d$; Agapow and Burt 2001) were calculated and tested using a permutation approach ($n = 1000$) and comparing the observed index of association with the expected index value that is independent of sample size. Since linkage disequilibrium can result from clonal reproduction, we calculated the number of clones in each population using the GenoDive program (version 3.06; Meirmans 2020). This was done by calculating the genetic distances between pairs of ramets and using a threshold of genetic similarity. Pairs of ramets falling below this threshold were considered clones. In this case, a threshold of zero (i.e., samples differing in one base pair were considering different clones) was used since it corresponded to the intermediate value between the first peak of frequency of genetic distances (due to possible errors from scoring or somatic mutations) and the second peak of frequencies (Meirmans and van Tienderen 2004). We also considered that errors during genotyping were unlikely, given the low number of alleles per loci we observed. Then, we did a diversity-based test to examine whether duplicated copies were due to asexual or sexual reproduction by randomizing alleles and evaluating the probability that the observed genetic diversity is lower than expected genetic diversity under random mating (the null-hypothesis is that they are similar; Gomez and Carvalho 2000). Lastly, we assessed whether loci had enough power to distinguish among unique genotypes by generating a curve of genotype accumulation using the "poppr" R package. The curve was calculated by doing a random resample ($n = 1000$) of loci and counting the number of genotypes observed. We also compared the number of unique genotypes present in South Africa to populations from the rest of the world. For these analyses we excluded the population from Cape Point (ZA8) due its very high genetic differentiation from all other populations and incidence of missing data at multiple loci (see Results section). The latter would inflate estimates of clonality.

At the population level, we calculated the number of alleles per locus (Na), number of effective alleles (Ne), Shannon's index (I), and observed and expected heterozygosity (H_O and H_E, respectively). To account for different sample numbers among populations, a rarefaction correction based on the smallest sample size (i.e., population CA8 with seven samples; Table 1) was applied. Further, we calculated the inbreeding coefficient (F_{IS}) for each population. All genetic diversity-related calculations were performed using GenAlex (version 6.5; (Peakall and Smouse 2012). We estimated the number of genotypes (G), number of effective alleles (Ne), Nei's (1987) genetic diversity corrected per sample size (H_e) and Nei's uncorrected genetic diversity (Gd) by using GenoDive.

Genetic structure and variation

To investigate the genetic structure among sampled populations, we performed Bayesian assignment tests, as implemented in STRUCTURE (version 2.3.4; Pritchard et al. 2000). We evaluated a range of possible genetic clusters (i.e., K values) by using an admixture model with correlated allele frequencies, 100,000 burn-in iterations, 1,000,000 Markov Chain Monte Carlo repetitions and 20 iterations for each value of K. To evaluate the optimum number of genetic clusters, we applied the delta K method described by Evanno et al. (2005) using the online software STRUCTURE HARVESTER (version 0.6.94; Earl and VonHoldt 2012). Each population was assigned to a specific genetic cluster when the average assignment values of all its individuals was >75% to that cluster. All remaining populations were considered to be admixed. We used the ade4 R package (version 1.7-22; Dray and Dufour 2007) to perform a principal coordinate analysis (PCoA) which was based on the uncorrected genetic distances (Cavalli-Sforza and Edwards 1967) calculated with FreeNA.

Morphology

Aiming to explore whether diagnostic morphological characters could help managers and other stakeholders identify invasive *Carpobrotus* species, we collected data on several morphological characteristics of 10 randomly chosen ramets per sampled population, many of which have been used by previous authors (Albert et al. 1997; Campoy et al. 2018). We selected one flower per ramet and recorded the colour of the petals (Fig. 2A) and filaments of the stamens (Fig. 2B), the shape of the receptacle (i.e., whether the receptacle had a V or U shape; Fig. 2C), the surface of the ovary (i.e., whether the ovary was raised, flat or depressed; Fig. 2D), the position of the calyx globes (i.e., whether the petals are longer, equal or shorter than the sepals; Fig. 2E), and the diameter of the flower (Fig. 2F) and the stamen ring (Fig. 2G). For each ramet, we also selected one leaf located at least two nodes below the apical leaf, and measured its total length, width, and thickness in the centre (Fig. 2H–J, respectively). Finally, we calculated the leaf cross section area as the leaf width, multiplied by the leaf thickness, divided by two.

We then built a regression tree using morphological characteristics as predictors and the genetic cluster to which each population was allocated as the response variable. We excluded those populations with admixed ancestry (Table 1). Regression trees were built using the classification method and pruned, choosing the best complexity parameter. We ran all regression tree analyses using the "rpart" package in R version 4.1.3 (Therneau and Atkinson 2017).

Data resources

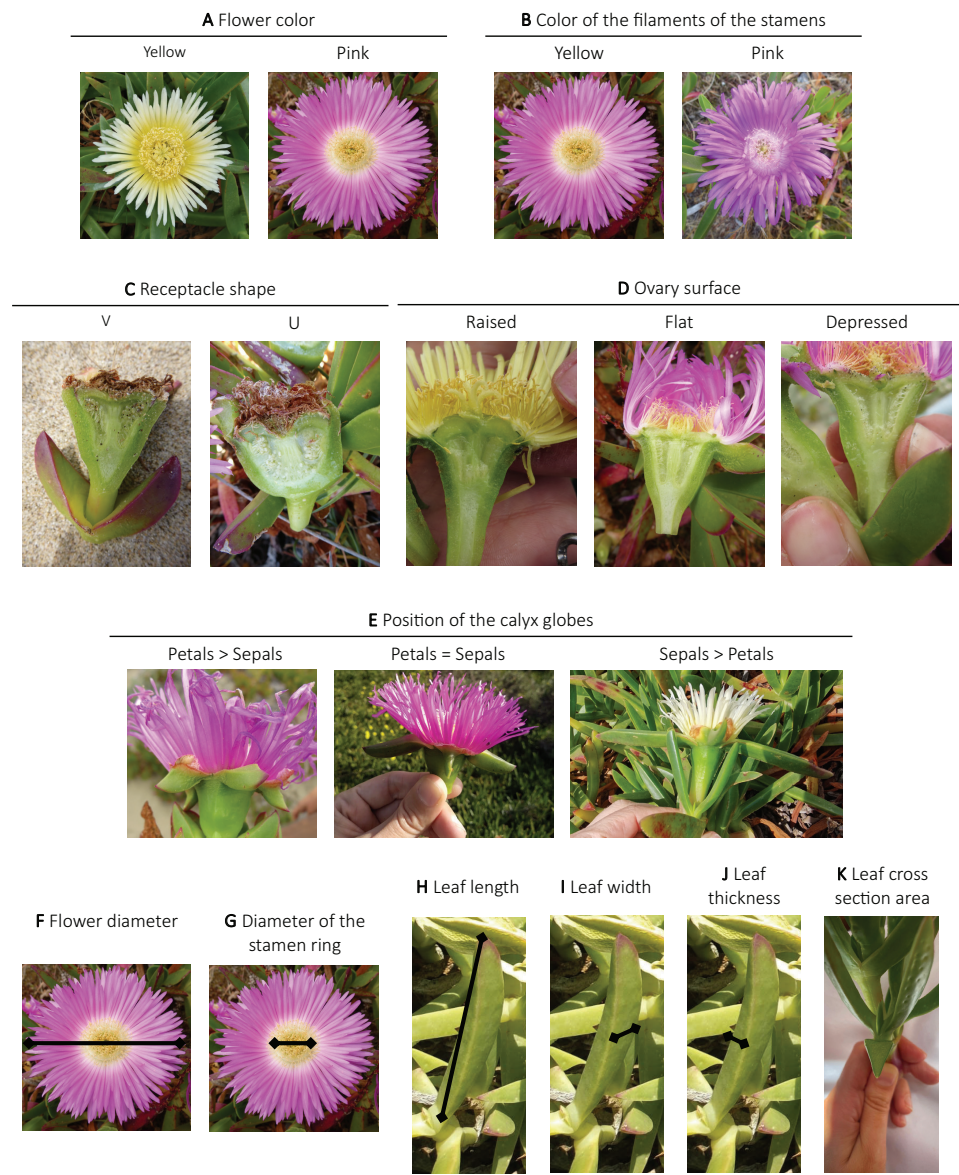The datasets generated during and/or analyzed during the current study are available in https://doi.org/10.5281/zenodo.8123272.

Figure 2. Morphological characteristics of *Carpobrotus* species measured in this study (see text for details).

Results

Dataset characteristics and genetic diversity

We found no evidence of scoring errors due to band stuttering in our genotype dataset. All six loci were polymorphic in the overall dataset and the number of alleles per locus ranged between two and nine.

Samples from Cape Point had a high incidence of missing data and were removed from subsequent analyses. This population likely represents a species that is distantly related to all other species we sampled in our study (average pairwise population F_{ST} = 0.7). For the remaining populations, we found the association index of alleles at different loci to be lower than expected in all populations, indicating the presence of linkage disequilibrium ($\bar{r}_d$ = 0.013; p > 0.001; Suppl. material 2). The GenoDive approach found clones within all populations, with one population from South Africa (Mdumbi) and California (Celestre), two populations from Azores (Ribeira Grande and San Vicente), three populations from Spain (A Lanzada, Punta de Rons and Samil) and one Argentinian population (Quequén) each containing genetically identical ramets (i.e., consisting of a single clone; Table 1). Only one South African population (Springfontein) did not have clones. The diversity-based test confirmed asexual reproduction for all populations except three from South Africa (Port Alfred, Port Elizabeth and Springfontein; Table 1). The genetic accumulation curve showed that the four loci were slightly deficient in distinguishing among genotypes (i.e., 201 of 204 unique multi-locus genotypes were identified after resampling). The maximum number of unique multi-locus genotypes was 204 for the 681 ramets sampled, thus ~70% of sampled ramets were clones. For South Africa, ~53% of sampled ramets were clones.

In all populations, *Na* was low (range 1.4–3.00). Observed heterozygosity was slightly higher (range 0.225–0.648) than H_E (range 0.131–0.444; Fig. 3), and F_{IS} values indicated that all populations have very low or no inbreeding. When comparing populations from the different sampled ranges, levels of H_O, H_E, and F_{IS} were similar.
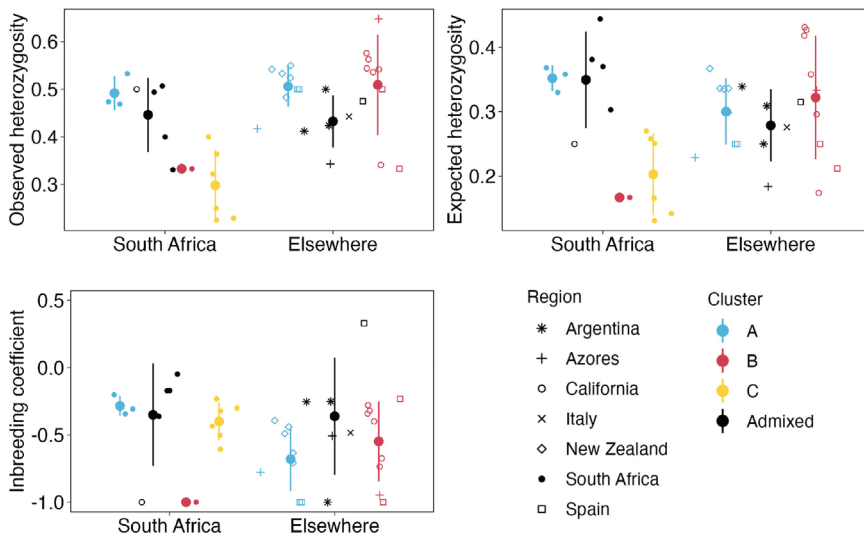
Figure 3. Genetic diversity metrics of native and non-native populations of *Carpobrotus* species. Colours indicate the cluster to which each population has been assigned (See Table 1 and Fig. 4 for further details). Although admixed populations are not necessarily genetically similar, they were combined for visualization purposes.

No differences in the level of these indices were found between population ranges of each cluster, except that H_O and H_E were lower in populations of cluster C (which were all South African populations) than in other populations (see below for genetic structure results, Table 1 and Fig. 4). For clonal diversity, *Ne* was low for most populations while *He* and *Gd* were relatively high.

Genetic structure

Population pairwise F_{ST} estimates (excluding population ZA8 from Cape Point) ranged from low ($F_{ST}= 0.015$; between populations ZA1 and ZA2) to high ($F_{ST}= 0.6$; between populations ZA5 and ZA10) (Suppl. material 1). The results of the STRUCTURE analysis, including 40 sampled populations identified $K = 3$ as the optimal number of genetic clusters (Fig. 4). Based on the criteria outlined in the Methods we identified 11 populations as being admixed, with the remaining populations having high overall assignment values to one of the three identified genetic clusters only. Although we found all three genetic clusters in South Africa, two of them were more dominant: cluster A along the west coast and cluster C along the east coast. In South Africa, only one population (Mdumbi) was clearly associated with cluster B (Fig. 5).
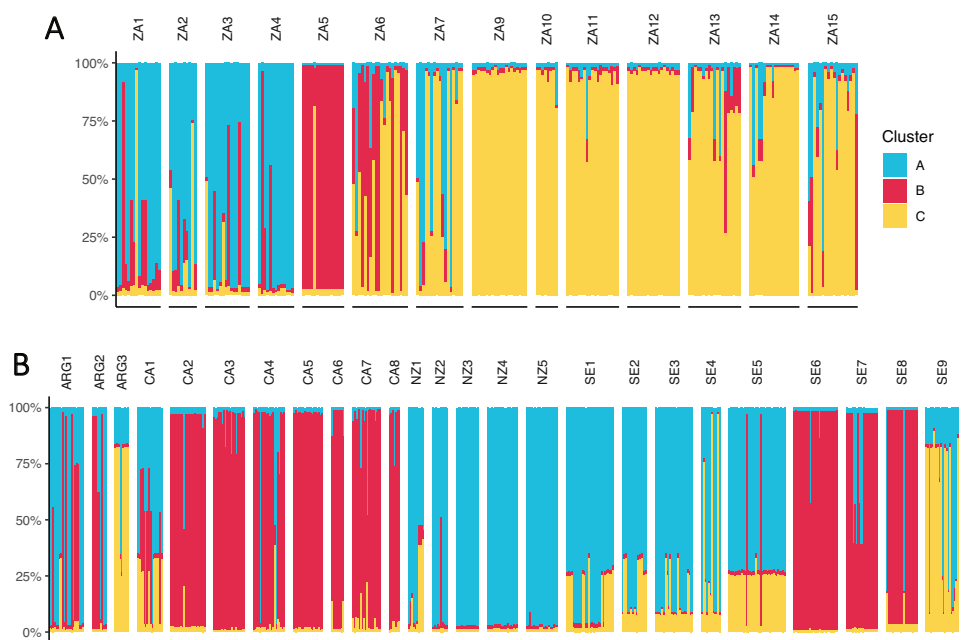
Figure 4. Bar plots showing the genetic structure of the **A** native South African and **B** non-South African populations of *Carpobrotus* species included in this study. Note that both plots represent the same analysis and were split into two panes for better visualization. The delta *K* method following Evanno et al. (2005) revealed $K = 3$ as the optimal number of genetic clusters. Abbreviations above the bar plots indicate the ID of the populations (see Table 1 for more details).
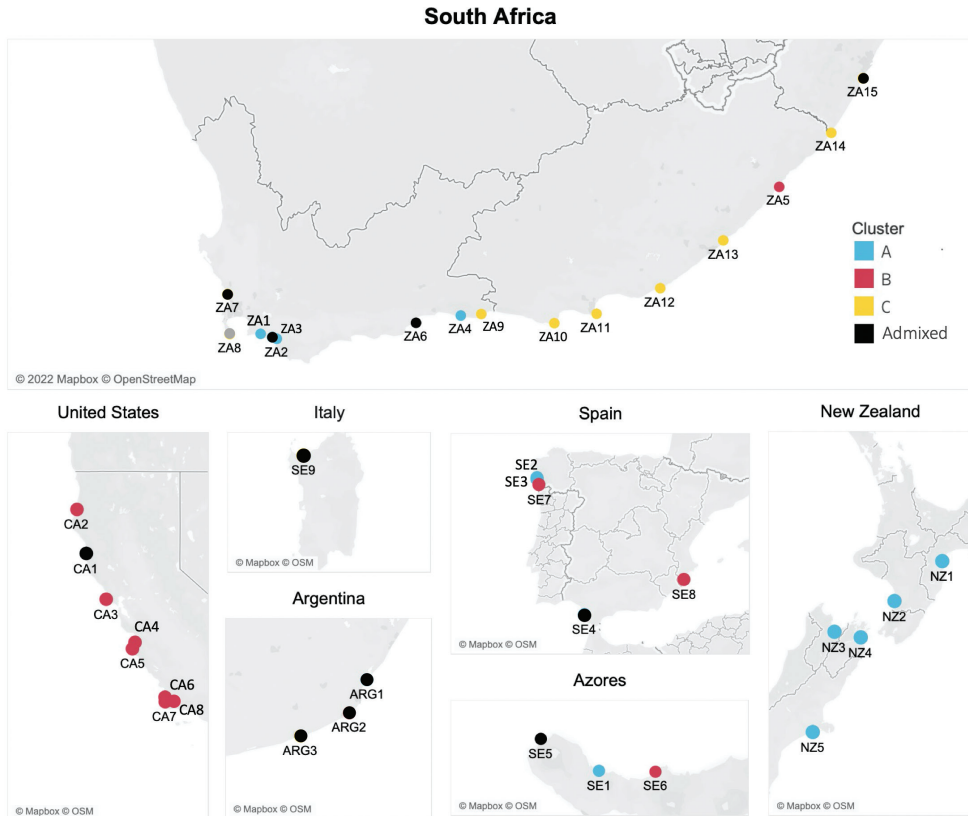
Figure 5. Geographical distribution of the populations of *Carpobrotus* species sampled in this study (see Table 1 for further details). Points roughly indicate the location of the populations. The colour of the points indicates the main cluster each population was assigned to (See Table 1 and Fig. 4 for further details). Cape Town population (ZA8) is indicated in grey since it had excessive missing data and, therefore, was removed from the analyses.

All five populations from New Zealand, two from Spain and one from the Azores (Portugal) appeared to be more closely related to west coast populations from South Africa (i.e., genetic cluster A). Six of the seven populations sampled in California (USA), together with one population from South Africa, two from Spain and one from the Azores (Portugal), formed a separate cluster (cluster B). A similar pattern was also observed in the PCoA, although one of the Spanish populations (i.e., SE8) was assigned to cluster B by the STRUCTURE analysis, and showed no clear association with any of the studied populations (Fig. 6). Moreover, the PCoA results showed one of the sampled Argentinian populations (i.e., ARG2) to be similar to those assigned to cluster B by the STRUCTURE analysis, while one population from Argentina (i.e., ARG3), one from California (i.e., CA1) three from Europe (i.e., SE4, SE5 and SE9) and one from South Africa (ZA2), previously classified as admixed, clustered with those assigned to cluster A.
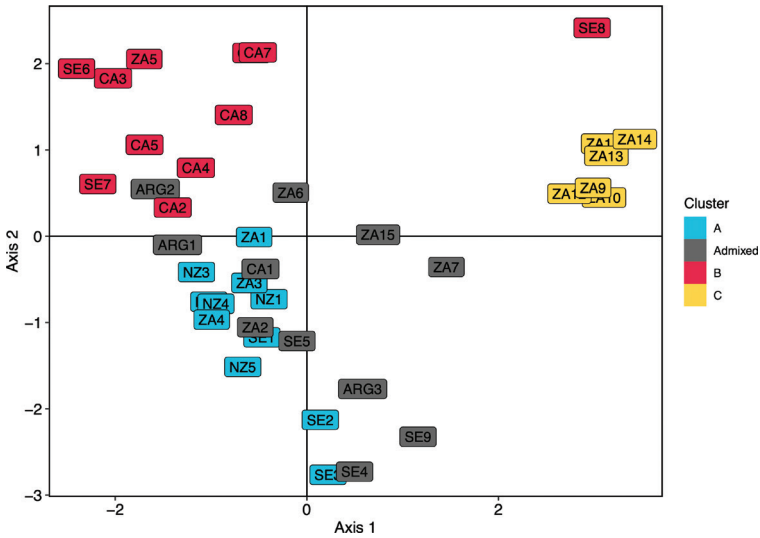
Figure 6. Principal coordinates analysis for the populations of *Carpobrotus* species included in this study. The analysis was based on genetic distances (following Cavalli-Sforza and Edwards 1967) between populations. Populations are indicated with different colours according to the main cluster they have been assigned to or levels of admixture (see Table 1 and Fig. 4).

Morphology

We found no clear link between morphological characteristics and the identified genetic clusters of *Carpobrotus* plants sampled in our study (Figs 7, 8). However, individuals belonging to cluster A generally present yellow stamens (62.8% of the sampled individuals), their sepals are longer or the same length than the petals (90.9%), and the diameter of their stamen ring is longer or equal to 2.1 cm (81%). Individuals assigned to cluster B always present yellow stamens, generally have a flat or raised ovary (95.3%), and their leaves are normally shorter than 10 cm (98%). The individuals assigned to cluster C have pink flowers, and generally present pink stamens (72%), generally have depressed ovaries (68%), their petals are generally longer or equal in length to their sepals (86%), and their stamen ring is generally smaller than 2.1 cm (78%). However, there were many exceptions to these patterns (Figs 7, 8).

Discussion

Our results confirm the complex identification, biosystematics and biogeography of the invasive *Carpobrotus* spp. The west coast of South Africa, and possibly California, were identified as the most likely sources of invasive populations worldwide.

The Bayesian assignment analysis grouped the sampled populations into three genetic clusters (clusters A, B and C; Fig. 3). In South Africa, the native distribution area of most *Carpobrotus* spp. (Germishuizen and Meyer 2003), most sampled individuals
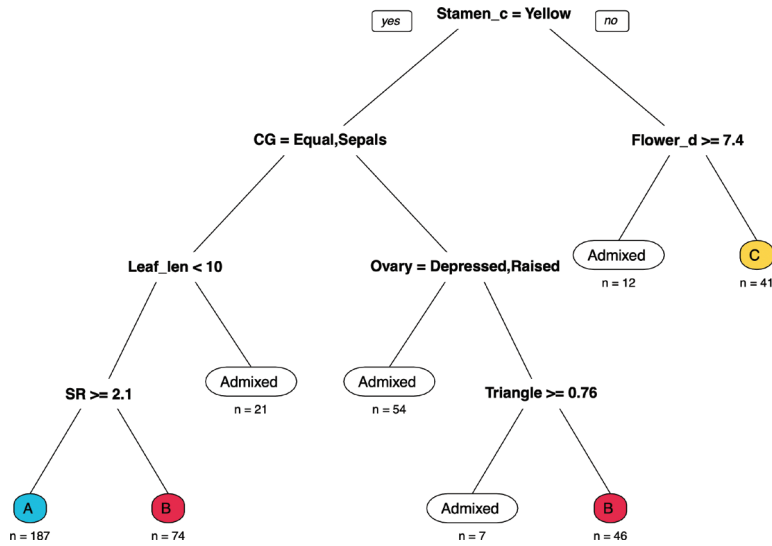
Figure 7. Classification tree analysis of the *Carpobrotus* genetic clusters based on morphological charac-
teristics. The most significant characteristic is indicated at each node, with the corresponding values relat-
ing to branches on the left. Morphological differences between genetic clusters could be best explained
by the color of the filaments of the stamens (Stamen_c), the flower diameter (Flower_d), the position
of the calyx globes (CG), the ovary surface (Ovary), the leaf cross section area (Triangle), the leaf length
(Leaf_len) and the diameter of the stamen ring (SR). Leaf_len, Flower_d and SR are indicated in cm.
Colours of circles at the end of branches correspond to the genetic clusters. *n* = number of individuals
assigned to each cluster. See Fig. 2 for details on the morphological characteristics included in the analysis.

were assigned to clusters A (in the Western Cape province) and C (in the Eastern Cape
and Kwazulu-Natal provinces). Four *Carpobrotus* species (including their described lower
taxa) are considered native to the Western Cape province: *C. acinaciformis*, *C. edulis*, *C.
muirii*, and *C. quadrifidus* (Smith et al. 1998). Individuals assigned to cluster A could
therefore correspond to one or several of these species, or to hybrids between them. On
the other hand, two species occur naturally in the Eastern Cape and KwaZulu-Natal
provinces: *C. deliciosus* and *C. dimidiatus* (Smith et al. 1998). Therefore, cluster C likely
corresponds to individuals of one or both of these two species or hybrids between them.

Only one South African population, consisting of a single genotype, was assigned to
cluster B (shared by some populations from southern Europe and California; Table 1).
This population is located in Mdumbi, a remote area that attracts tourists from all
over the world due to the presence of various ecotourism establishments and surfing
lodges (Hitchcock 2014). Cluster B was predominantly found in California, although
two populations from Spain and one in the Azores were also from this cluster. These
results suggest that populations assigned to cluster B might have originated from South
Africa decades ago, introduced to California directly from South Africa or secondarily
via Spain (i.e., a country with an extensive history of trade with the California coast;
Engstrand 1997), and hybridized extensively (Vilà et al. 1998; Schierenbeck et al.
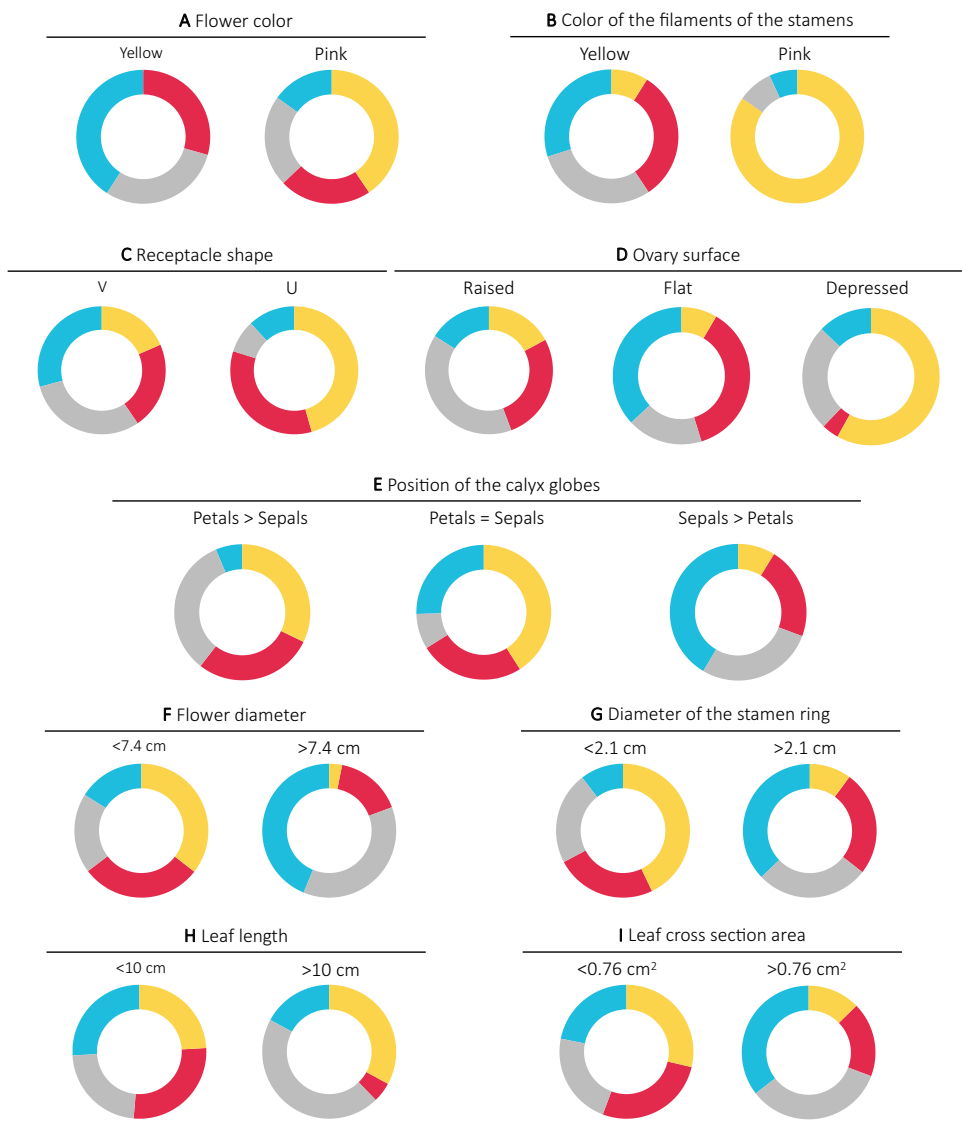
Figure 8. Donut plots representing the proportion of *Carpobrotus* individuals belonging to different genetic clusters, or admixtures between them, and morphological characteristics (see Fig. 2 for details). Blue = cluster A; red = cluster B; yellow = cluster C; grey = admixed.

2005) with *C. chilensis*, a species of unknown origin that mainly occurs in the Pacific coasts of the American continent (Campoy et al. 2018). The abrupt appearance of *C. chilensis* pollen within a 900-year-old record for the central California coast in the early 1800s suggests introduction with early Spanish settlement or visitation including extensive migration of people from Portugal from the Azores to coastal California in the 1800s (Williams 1982; Bicknell and Mackey 1988). Individuals representing cluster B

may then have been introduced to the coast of Spain and/or the Mdumbi region after hybridization had occurred with the later introduced *C. edulis*. It is also possible that the *C. chilensis* plants in California arose from an early introduction from an unknown source where the species no longer exists and then pure *C. chilensis* in California has largely disappeared through hybridization.

Outside South Africa and California, all sampled populations were assigned to either cluster A or B, or were identified as admixed. These findings suggest that the Western Cape province of South Africa and coastal California may have served as the sources for many introduced *Carpobrotus* populations in the rest of the world. This is not surprising, given that *Carpobrotus* species have been widely introduced as ornamental plants (Campoy et al. 2018), and both regions have been prominent hubs of the ornamental horticulture industry for centuries (University of California 1999). More specifically, all individuals sampled in New Zealand were assigned to cluster A, suggesting a South African origin of *Carpobrotus* invasions in this country. In Spain and Azores, most populations were assigned to clusters A and B, suggesting multiple introductions from South Africa and the Americas.

The Italian and Argentinian populations included in our analyses were not clearly assigned to particular genetic clusters, suggesting that genetically distinct groups or species of *Carpobrotus* were introduced to these areas from different sources, leading to extensive admixture (Suehs et al. 2004). Accordingly, hybridization has been repeatedly suggested to play an important role in the invasiveness of *Carpobrotus* species (Campoy et al. 2018), with hybrids having higher survival and faster growth rates than parental taxa (Vilà and D'Antonio 1998). Moreover, our results show that hybridization is also common in South Africa, the native range of most species in the genus. The implications of hybridization for the invasion of *Carpobrotus* are poorly understood and deserve further research attention.

Overall, our results indicate that there have been multiple introductions of *Carpobrotus* species from different sources globally. Typically, multiple introductions increase the genetic diversity and probability of success of invasive species (Genton et al. 2005; Walls 2010). However, we found extremely low levels of genetic diversity in all studied populations. The reason for this can probably be attributed to the capacity for self-fertilization (Vilà et al. 1998) and the clonal nature of *Carpobrotus* species, which facilitates vegetative reproduction without genetic recombination (Campoy et al. 2018) which typically results in low genetic diversity (e.g., Hollingsworth and Bailey 2000). Accordingly, we observed low inbreeding and high clonality levels in all sampled populations. Moreover, clonality has been suggested to allow *Carpobrotus* species, and alien plants in general, to effectively establish and colonize new areas (Roiloa et al. 2010). These observations also explain the high number of monomorphic loci we identified during genetic marker development and testing.

Accurate identification of invasive *Carpobrotus* species or hybrid combinations could improve risk assessment and guide early detection and rapid response management actions (Guisan and Thuiller 2005). For example, in California, some managers do not want to remove what seems to be *C. chilensis* because they do not know whether

or not it is native and it appears to coexist with native species and can be helpful in dune stabilization (D'Antonio, personal observation). Also, "taxonomic identity" should be specified in any risk assessment/analysis scheme (e.g., IPPC, ISPM 2, Framework for pest risk analysis) and local management plans for the removal of species. Similarly, Species Distribution Models (SDMs) used to guide early detection and rapid response actions typically use distributional data of the target species, coupled with characteristics of the current and potentially suitable areas (e.g., climate, land-use type) (Guisan and Thuiller 2005). Using Ecological Niche Models, Thuiller et al. (2005) identified areas of high suitability for invasive *Carpobrotus* species in Australia, central east Africa, Chile, Europe and the USA. However, it is conceivable that the geographic extent of such predictions depends on the occurrence records of the *Carpobrotus* species and/or their hybrids used to calibrate these models. Accurate identification and knowledge of the introduction history of invasive *Carpobrotus* spp. are also critical for reducing the negative impacts of their current invasions. The most common methods used to control *Carpobrotus* invasions include mechanical and chemical methods (Ruffino et al. 2015). However, these methods require large amounts of funding and capacity, follow-ups and restoration efforts, and have not been successful at reducing *Carpobrotus* invasions at large geographic scales. The integration of biological control into the management of invasive *Carpobrotus* species could reduce management costs significantly and increase management success (Campoy et al. 2018). For example, the South African soft scale *Pulvinariella mesembryanthemi* (Vallot, 1829) is a specialist herbivore of *Carpobrotus* spp. that was accidentally introduced into California, causing considerable damage to invasive populations of *C. edulis* (Washburn and Frankie 1985) where it also became a pest of the presumed native *C. chilensis* (Schmalzer and Hinkel 1987). Subsequently, predators and parasites were released from South Africa to control the scale (Tassan et al. 1982). *Pulvinariella mesembryanthemi* is still a promising potential biological control agent outside of California (Vieites-Blanco et al. 2019; Núñez-González et al. 2021). But the effectiveness of *P. mesembryanthemi* is likely to depend on the taxonomic identity and source region of the target species (Pyšek et al. 2013; Le Roux 2021).

However, identifying invasive *Carpobrotus* species is challenging. Several diagnostic morphological characters have been proposed to differentiate between species, with petal colour being the most popular one (Preston and Sell 1988; Wisura and Glen 1993), but doubts have been expressed on the validity of all proposed characters as taxonomic markers (Campoy et al. 2018). Our results show no clear pattern regarding the association of morphological traits with the three genetic clusters we identified. We only collected morphological data from 10 individuals per population in the field, and each population was located in a different coastal habitat (e.g., disturbed areas or dunes). The different conditions to which *Carpobrotus* individuals were exposed in the field might have added a large variation to our morphological results. Additionally, within some populations, variation between individuals was high, potentially swamping differences across populations. Moreover the widespread occurrence of hybrid populations makes identification using morphological data even more difficult (Suehs et al. 2004).

Despite the challenges related to the morphological identification of invasive *Carpobrotus* species using morphological characters, our results have important implications for the development of management programmes. First, no introductions of individuals from cluster C have been detected in any of the sampled sites. However, the rate of introduction of alien species is rapidly increasing (Seebens et al. 2021). This, coupled with the widespread use of *Carpobrotus* species as ornamental plants, enhances the chances of individuals from cluster C to be introduced and the potential for genetic exchange between populations from all three clusters, which could increase the invasion success of *Carpobrotus*. Hence, management strategies should aim at preventing the introduction of additional *Carpobrotus* genotypes, especially from the Eastern South African coast. Second, we revealed that the most probable sources of *Carpobrotus* introductions and invasions globally are the Western Cape province in South Africa and California. Since most effective biocontrol agents are generally those that have co-evolved with the invasive species (Müller-Schärer et al. 2004), the search for biocontrol agents to manage *Carpobrotus* invasions should be focused in these areas. A challenge for this in California is the fact that pure *C. chilensis* is rare due to the extensive hybridization, and no specialist insects have been observed on it other than the rare occurrence of the introduced scale insects *Pulvinariella mesembryanthemi* and *Pulvinaria delottoi* (Schmalzer and Hinkel 1987). Moreover, there is no clear evidence that *C. chilensis* is native to California, and future studies should extend sampling efforts to other areas such as the coasts of Chile and Australia. Additionally, it should be carefully explored whether biocontrol agents from the Western Cape province in South Africa and California are effective at managing admixture (or hybrid) populations or *Carpobrotus* invasions in general.

Our work highlights exciting opportunities for future research on *Carpobrotus* invasions. For example, high-resolution population genomic analyses (e.g., single nucleotide polymorphism genotyping or whole genome sequencing), coupled with common garden experiments, would provide valuable insights into the diversity and evolutionary dynamics of the genus, the invasiveness of its representatives and their interactions with insects with the potential to be used for biological control. For instance, a highly flexible breeding system that allows extensive hybridization (i.e., outcrossing) and high levels of clonal reproduction (via vegetative structures) suggest the stabilization of highly successful hybrid genotypes is likely to occur. Determining whether certain hybrid combinations and/or clones are more prevalent in native or invasive ranges should be included in future research to inform future management of the group.

Acknowledgements

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Supplementary material 1

Pairwise estimates of genetic differentiation based on microsatellite data (FST)

Authors: Ana Novoa, Heidi Hirsch, María L. Castillo, Susan Canavan, Luís González, David M. Richardson, Petr Pyšek, Jonatan Rodríguez, Lurdes Borges Silva, Giuseppe Brundu, Carla M. D'Antonio, Jorge L. Gutiérrez, Megan Mathese, Sam Levin, Luís Silva, Johannes J. Le Roux

Data type: xlsx

Explanation note: Pairwise estimates of genetic differentiation based on microsatellite data (FST) for all populations included in this study. Note that 'population ID' refers to the same IDs provided in Table 1 in the main manuscript.

Link: https://doi.org/10.3897/neobiota.89.109164.suppl1

Supplementary material 2

Testing for linked disequilibrium on 681 samples of *Carpobrotus* species

Authors: Ana Novoa, Heidi Hirsch, María L. Castillo, Susan Canavan, Luís González, David M. Richardson, Petr Pyšek, Jonatan Rodríguez, Lurdes Borges Silva, Giuseppe Brundu, Carla M. D'Antonio, Jorge L. Gutiérrez, Megan Mathese, Sam Levin, Luís Silva, Johannes J. Le Roux

Data type: pdf

Explanation note: The blue line ($\bar{r}_d$) indicates the expected index of association of alleles at different loci that is independent of sample size. A distribution below $\bar{r}_d$ indicates linkage disequilibrium and evidence for clonal reproduction.

Link: https://doi.org/10.3897/neobiota.89.109164.suppl2

RESEARCH ARTICLE

The devil is in the details: exploring how functionally distinct round goby is among native fish in the Baltic Sea

Antoni Vivó-Pons[1], Isa Wallin-Kihlberg[2], Jens Olsson[2], Peter Ljungberg[2], Jane Behrens[3], Martin Lindegren[1]

1 *Centre for Ocean Life, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark, Kemitorvet, Building 202, 2800 Kongens Lyngby, Denmark* **2** *Swedish University of Agricultural Sciences, Department of Aquatic Resources, Institute of Coastal Research, Skolgatan 6, 742 22 Öregrund, Sweden* **3** *Section for Marine Living Resources, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark, Kemitorvet, Building 202, 2800 Kongens Lyngby, Denmark*

Corresponding author: Antoni Vivó-Pons (avipo@aqua.dtu.dk)

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Abstract

Understanding the characteristics and conditions that make non-indigenous species (NIS) successful at establishing in recipient communities is a key in determining their potential impacts on native species, as well as to improve management actions such as prevention of future invasions. The round goby (*Neogobius melanostomus*) is one of the most widespread non-indigenous fish species in the Northern Hemisphere, including the coastal zones of the Baltic Sea. The impacts of round goby in the Baltic Sea are pronounced and multifaceted, yet our knowledge regarding the underlying assembly processes determining its establishment is limited. To overcome this knowledge gap, we applied a trait-based approach to assess the degree of niche overlap and functional (trait) similarity between round goby and native fish species in coastal areas from the Baltic Sea, based on the functional distinctiveness metric. Our results show that round goby is generally quite similar (or not dissimilar) to the native fish of the regional species pool, at least in terms of its overall trait composition. Conversely, round goby demonstrates pronounced differences compared to the native community in its display of parental care and territorial behaviour. Such differences in individual traits could play an important role in round goby's invasion success in the Baltic Sea, including its interactions with native species (e.g. competition). Our results and their potential implications may be highly relevant for conservation and management if integrated within existing risk assessment tools for biological invasions in order to prioritise and enhance the effectiveness of preventative actions towards the expansion of round goby.

Introduction

The introduction and spread of non-indigenous species (NIS) constitute a major threat to global biodiversity, ecosystems and their associated services (Bax et al. 2003; Millennium Ecosystem Assessment (Program) 2005; IPBES 2023). In marine ecosystems, the spread of NIS has increased during the last decades, largely due to increased global marine transport (Vitousek et al. 1996; Rilov and Crooks 2009). On a global scale, the impacts of such introductions are negative and may interact in a synergistic or additive way with other anthropogenic impacts (Anton et al. 2019; Geraldi et al. 2020). Yet, at local spatial scales, the effects of NIS can be highly diverse and context-dependent and even include neutral or positive effects (Katsanevakis et al. 2014; Viana et al. 2019; Vivó-Pons et al. 2020).

Before having an impact on native communities, NIS need to be successfully established in the recipient area with self-sustaining populations (Blackburn et al. 2011). The establishment depends on several community assembly processes (Gallien et al. 2015; Kraft et al. 2015; Montanyès et al. 2023) related to both abiotic and biotic factors. In communities heavily influenced by abiotic factors, species are expected to be functionally alike, with a common set of traits to be able to cope with the environmental conditions (Zobel 1997). Conversely, in communities mainly shaped by biotic factors, such as competition (i.e. limiting similarity), species tend to be functionally dissimilar or occupy more specialised niches (Gallien et al. 2014). Despite the conceptual understanding of NIS and the role of community assembly rules affecting their establishment, our empirical insight regarding the degree of niche overlap (i.e. functional similarity/dissimilarity) of NIS and native species is limited (Gallien and Carboni 2017), especially in marine ecosystems. For instance, it is debated whether NIS generally display similar or dissimilar traits compared to native species of recipient communities (Gallien and Carboni 2017) and to what degree the similarity or dissimilarity of NIS may affect their invasion success. Previous studies suggest that NIS both can establish by being functionally similar to natives (Cleland 2011; El-Barougy et al. 2020) or by being dissimilar to natives (Ricotta et al. 2010; Escoriza and Ruhí 2016; Mathakutha et al. 2019; Steger et al. 2022; Xu et al. 2022). Thus, disentangling the different assembly processes shaping communities and the niche overlap between NIS and native species' niches is fundamental to better understand biological invasions and their associated impacts on native communities and ecosystems (Ricciardi et al. 2013).

The Baltic Sea is one of the largest brackish water bodies in the world, demonstrating a pronounced north-south salinity gradient from fully marine- to almost freshwater conditions in the northern parts (Voipio 1981; HELCOM 2018). Due

to shipping and man-made waterways, the Baltic Sea contains 173 recorded NIS, many of which display self-sustaining populations (Leppäkoski et al. 2002; Ojaveer et al. 2010, 2017; Reusch et al. 2018; ICES 2022a) and whose introduction events have become more pronounced in recent decades (HELCOM 2023). The susceptibility of the Baltic Sea to the introduction and establishment of NIS is likely due to a combination of anthropogenic disturbances (e.g. eutrophication, pollution, intensive fishing and climate change), as well as the naturally low biodiversity and its brackish water conditions, allowing NIS of both marine and limnetic origin to settle and establish (Paavola et al. 2005; Ojaveer et al. 2010; Olenin et al. 2017; Reusch et al. 2018).

The round goby (*Neogobius melanostomus*), originally from the Ponto-Caspian area, is one of the most widespread invasive fish species in the Northern Hemisphere (Kornis et al. 2012; ICES 2022b). In the Baltic Sea, it was first detected in the Gulf of Gdansk in 1990 (Skóra and Stolarski 1993) and has since then established and spread in most coastal zones of the region (ICES 2022b), where secondary spread has likely been aided by shipping (Azour et al. 2015; Kotta et al. 2016). The invasion success of this species has been manifested by rapid population growth in recent years (Kruze et al. 2023), with densities occasionally reaching 20 individuals/m^2 (Puntila-Dodd et al. 2018). Round goby feeds on a wide range of prey (Schwartzbach et al. 2020; van Deurs et al. 2021; Wallin-Kihlberg et al. 2023), displays aggressive behaviour (Dubs and Corkum 1996, Balshine et al. 2005, Ericsson et al. 2021), is tolerant to a wide range of temperatures and salinities (Behrens et al. 2017, 2022; Christensen et al. 2021) and has a high reproductive turnover rate (Jude 1997). Although the overall impacts of its establishment are deemed ecosystem- and context-specific (Hirsch et al. 2016), it has been shown to decimate local invertebrate populations (van Deurs et al. 2021; Nõomaa et al. 2022), compete with native species for prey (Karlson et al. 2007; Skabeikis et al. 2019; Ericsson et al. 2021) and create new energetic pathways (Almqvist et al. 2010). Thus, the impacts of round goby on Baltic Sea food webs and habitats are pronounced and multifaceted, yet our knowledge regarding the underlying assembly mechanisms and processes determining its establishment is still limited.

Trait-based approaches provide a mechanistic way to address key aspects of biological invasions (Violle et al. 2007; Belmaker et al. 2013; Quell et al. 2021 Steger et al. 2022 Vivó-Pons et al. 2023). This is because traits of NIS may highlight potential interactions and niche overlap with native species and also contribute to the understanding of community assembly processes determining NIS establishment. In this study, we use a trait-based approach to investigate the degree of niche overlap and functional distinctiveness of round goby relative to native fish species in the recipient communities using the Baltic Sea as a case study. We aimed to address the following questions: i) Is round goby functionally distinct or similar compared to the regional pool of native species? ii) Which traits make round goby more or less distinct? iii) To what extent is the distinctiveness of round goby at the local scale influenced by abiotic and biotic factors?

Materials and methods

Data collection

Monitoring data of coastal fish communities where round goby is present was obtained from the Swedish national and regional coastal fish monitoring programme as registered in the national coastal fish database - KUL (https://www.slu.se/kul). The data were extracted for 14 locations sampled between 2008–2021, covering from the southwestern Baltic Sea (Stavstensudde) to the Bothnian Sea (Gävlebukten; Fig. 1). Two gear types, Nordic coastal multi-mesh monitoring gillnets and sets of nets, were used in the selected monitoring locations. The Nordic nets consist of nine panels of different mesh-sizes (10, 12, 15, 19, 24, 30, 38, 48 and 60 mm centre knot-to-centre knot), are 1.8 m deep and 50 m long. The sets of nets consist of a number of linked mono-mesh gillnets with mesh sizes between 21–60 mm centre knot-to-centre knot (HELCOM 2019). In Mönsteras, Simpevarp, Muskö and Vinö, between four and six stations were fished over three to six nights within the same week every year with the sets of nets. In the rest of the monitoring locations, between 35 and 50 fixed stations were fished with Nordic nets during one night per year, within the same week every year. Those stations are distributed according to depth-stratified design covering 0–3, 3–6, 6–10 and 10–
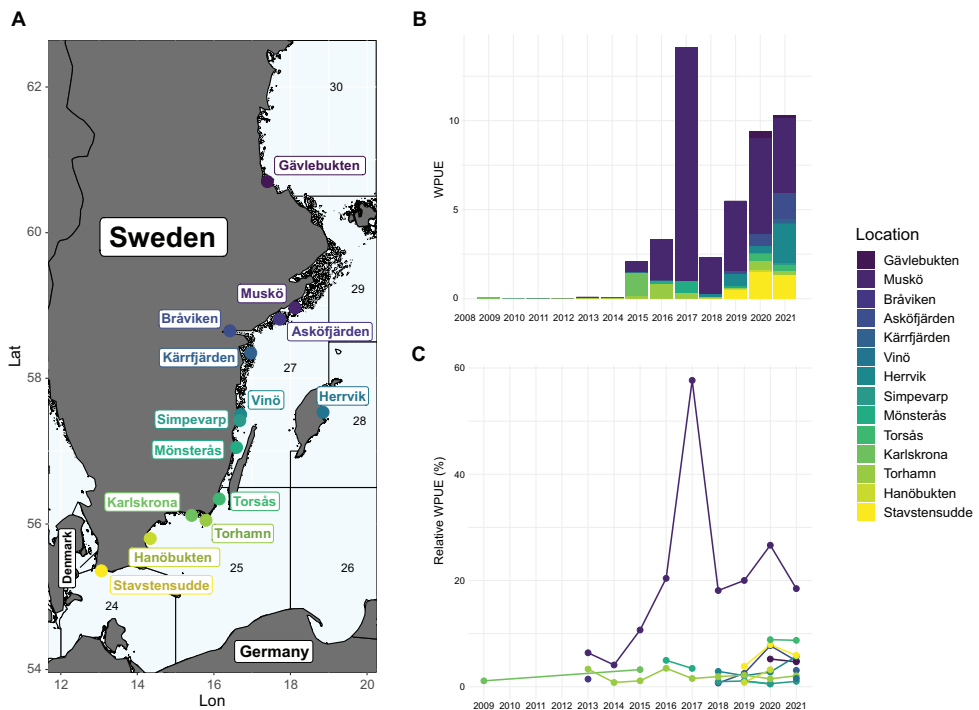

Figure 1. Map of the study area including positions of all sampled locations (**A**). WPUE of round goby for each year and sampling location (**B**). Mean relative WPUE of round goby per year at each location (**C**).

20 m depths (HELCOM 2019). Both types of gears used are set at the bottom of the coastal area. All catches were registered as numbers of individuals per species per length class (1 cm). Thereafter, the total weight per species (g) was transformed into weight per unit effort (WPUE). As not all fish were weighed during the monitoring, weight-length relationships were used to estimate the species weight if missing. It is important to note that the gears used are not the most optimal to catch round gobies or other demersal species with a more sedentary behaviour. Hence, the derived relative biomass (WPUE) of round goby compared to the native species is likely underestimated. To avoid inclusion of sporadically occurring species, we only included those species representing 99.5% of the total species occurrences in the data, resulting in a total of 27 species. We separated the total initial weight identified as *Platichthys flesus* in our data, based on observed proportions between *P. flesus* and *P. solemdali*, a recently discovered cryptic species of flounder (Momigliano et al. 2018; Florin et. al. unpublished data).

Trait data collection

In order to represent the general ecology of the species, a total of 11 categorical traits were selected, with 37 different trait modalities: habitat switching, parental care, territorial behaviour, diet, temperature preference, development mode, pharyngeal bones, habitat, fin type, body type and length class (Table 1). The classification and selection of traits builds on previous trait-based descriptions of marine organisms aiming to represent their behaviour, feeding, reproductive or survival strategies (Litchman and Klausmeier 2008; Törnroos and Bonsdorff 2012; Litchman et al. 2013). Similar criteria for trait selection have been adopted in recent studies of marine fish community structure and changes (e.g. Dencker et al. (2017); Pecuchet et al. (2017); Beukhof et al. (2019a, 2019b)). Trait information was obtained from online trait data portals like FishBase (https://www.fishbase.org.au/v4), scientific and grey literature and, when needed, supplemented by expert knowledge, following the same procedure as in Törnroos et al. (2015, 2019). Traits where species display a single modality (e.g. territorial behaviour, fin type, body type) were treated as categorical. For multi-choice nominal traits, where species can display multiple modalities (e.g. diet), each modality was scored between 0 and 1 representing the probability of being displayed by a given species (Suppl. material 1: table S1). For example, a species feeding mainly on benthic prey, but that occasionally feeds on other fishes, could receive a score of 0.8 for the modality "benthivorous" and 0.2 for the modality "piscivorous". Otherwise, if a species feeds equally on benthic and fish prey, it would be scored 0.5 for each modality.

Functional distinctiveness between round goby and native species

To assess the degree of (trait) niche overlap between round goby and native species, we used the functional distinctiveness index (D), weighted by species biomass. The functional distinctiveness index is defined as the mean functional distance of a single species to all other species present in a given community (Violle et al. 2017):

$$D_i = \frac{\sum_{j=1;j\neq i}^{N} d_{ij} \times Ab_j}{\sum_{j=1;j\neq i}^{N} Ab_j} \qquad \text{(Equation I)}$$

where d_{ij} is the functional distance between species i and j, N accounts for the number of species in the community and Ab_j accounts for the relative importance (i.e. relative WPUE) of species j. A high D value indicates that a species is functionally distinct compared to the other species in the community (Violle et al. 2017).

We computed the functional distance between each pair of species (d_{ij}) given by Gower's general coefficient of similarity (Gower 1971). This dissimilarity metric represents functional distances by giving equal weights between traits coded in different format (i.e. numerical, categorical or ordinal; Pavoine et al. (2009)). We are aware that mixing continuous with categorical, dummy or fuzzy-coded traits could result in a bias in the computed dissimilarities, due to an unbalanced contribution of traits coded in non-continuous formats (de Bello et al. 2021). However, in our analysis, we do not have any continuous traits (Table 1; Suppl. material 1: table S2) and, therefore, this potential issue is not directly applicable to our study. In any case, to avoid bias due to one or a few traits having a disproportional effect on D, we used an integrated process testing for multiple combinations of traits to compute the functional distances between species, obtaining a single distances matrix for each possible combination of traits (Coulon et al. 2023; Vivó-Pons et al. 2023). The resulting matrices obtained from all the possible trait combinations were summarised into a mean functional distance matrix for each pair of species present in the regional pool. From this overall mean functional distances matrix, we computed functional distinctiveness for all species, including round goby, both at a regional and local spatial scale with the corresponding regional or the different local species pools. To weight distinctiveness at a regional scale we obtained a unique value representing the overall relative WPUE of each species from 2009 to 2021, in order to cover the whole invasion process of round goby from the initial occurrence in the region to its subsequent spread. At a local scale, round goby's distinctiveness was weighted using the exact relative WPUE at each unique sampling event, i.e. within each sampled community. The functional distances were computed with the function "compute_dist_matrix" from the "funrar" package (Grenié et al. 2017) in R software, version 4.1.0 (R Core Team 2021).

In order to investigate if round goby was more or less distinct than the other species in the regional pool, we compared the value of functional distinctiveness of round goby relative to the values for all native species in the data set. Furthermore, to assess and compare the degree of niche overlap in trait space between round goby and the native species, we performed a Principal Coordinate Analysis (PCoA) on the overall pairwise dissimilarity matrix for the regional species pool (Belmaker et al. 2013; Vivó-Pons et al. 2023). Subsequently, we classified the species as dissimilar or similar, based on their distinctiveness value by grouping them into quartiles. The first quartile accounted for the functionally common species, while the fourth quartile accounts for the most functionally distinct species with higher values of distinctiveness.

Table 1. List of included traits and modalities, the number and percentage of species displaying each modality and the explanation of each modality. Modalities in bold are displayed by round goby.

Traits	Nature of the trait	Categories (n = 37)	N species having that category	Frequency (% of species having that category)	Explanation
Diet	Multichoice nominal	**Benthivorous**	18	46.22	Feeding mainly on benthic invertebrates as adults
		Planktivorous	7	16.59	Feeding mainly on plankton as adults
		Generalist	7	21.59	Feeding on the other categories as well as on detritus, algae etc. as adults
		Piscivorous	8	15.56	Feeding mainly on fishes as adults
Habitat	Categorical	**Demersal**	16	59.26	Living and feeding on or near the bottom as adults
		Benthopelagic	7	25.93	Living and feeding near the bottom as well as in mid-waters or near the surface as adults
		Pelagic	4	14.81	Living and feeding in the open water throughout ontogeny
Fin type	Categorical	Emarginated	5	18.52	Caudal fin with a rather sharp and straight end with an indent in the middle
		Forked	12	44.44	Caudal fin with the indent deeper than in emarginated fins
		Absent	1	3.70	
		Rounded	8	29.63	Caudal fin evenly rounded and convex
		Truncated	1	3.70	Caudal fin with a rather sharp edge that can be flat, square or straight
Body type	Categorical	Deep	6	22.22	Body is compressed from the sides
		Elongated	8	29.63	Body is rather long and slender
		Flat	2	7.41	Body is flat (depressed) with eyes on the same side
		Normal	11	40.74	Body is proportional and neither compressed nor depressed
Development mode	Categorical	Scattered	4	14.81	Eggs are scattered on the bottom
		Viviparous	1	3.70	Eggs receive nourishment from the female during development and hatch inside the body of the female
		Ovoviviparous	1	3.70	Eggs do not receive nourishment from the female during development
		Pelagic	2	7.41	Eggs float freely in the water column
		Adherent	17	62.96	Eggs adhere to a substrate in a layer
		Mass clump	2	7.41	Eggs adhere to each other, forming a clump
Length class (maximum length according to FishBase)	Ordinal	0–10 cm	2	7.41	
		10–20 cm	11	40.74	
		21–30 cm	7	25.93	
		31–40 cm	2	7.41	
		41–50 cm	5	18.52	

Traits	Nature of the trait	Categories (n = 37)	N species having that category	Frequency (% of species having that category)	Explanation
Temperature preference	Categorical	Cold	9	33.33	
		Warm	18	66.67	
Territorial behaviour	Categorical	**Yes**	8	29.63	The species holds and defends a territory or has a very narrow home range, usually related to spawning, but not necessarily
		No	19	70.37	
Parental care	Categorical	**Yes**	8	29.63	The species exhibits some sort of parental care, for example, carries or guards the eggs/young
		No	19	70.37	
Habitat switching	Categorical	**Yes**	21	77.78	The species switches habitat due to spawning, feeding migration or winter migration
		No	6	22.22	
Pharyngeal bones	Categorical	**Yes**	16	59.26	The species has pharyngeal bones or branchial tooth plates
		No	11	40.74	

Key traits affecting distinctiveness

To assess the effect and relative importance of each trait on functional distinctiveness, we calculated the difference between the distinctiveness values for each species based on all traits (Di, T) and the values when each individual trait was removed from the analysis (Di, T-t). We then divided the difference by regional distinctiveness, including all traits (Di, T) as follows:

$$Effect\ of\ trait_t\ on\ D_i = \frac{D_{i,T} - D_{i,T-t}}{D_{i,T}} \times 100 \qquad \text{(Equation II)}$$

Drivers of round goby functional distinctiveness

In order to reflect the key environmental conditions affecting the local distinctiveness of round goby at each sampling site, we compiled data of bottom salinity, temperature and depth, measured in situ as part of the fish monitoring programme. For some locations, bottom salinity and temperature data were incomplete, hence we complemented the monitoring programme data with data derived from the ice-ocean model NEMO-Nordic (based on NEMO-3.6, Nucleus for European Modelling of the Ocean; https://doi.org/10.48670/moi-00013) from Copernicus Marine Service (https://marine.copernicus.eu/). Before completing the available in-situ data with model-derived data, we compared values of available environmental variables derived from both sources. This sensitivity test showed a high correlation for both bottom temperature (r = 0.75) and salinity (r = 0.77) (Suppl. material 1: fig. S1). Therefore, we decided to use both in-situ data and modelled data for sampling events lacking such information. In addition, we also obtained the model derived data on dissolved oxygen and chlorophyll

a for each location at the corresponding sampling date. We used distance to the open sea as a proxy for coastal exposure, extracted by using the "cost distance" function in ArcGIS Pro (see Erlandsson et al. (2021) for details). Then, to estimate the effect of biotic drivers acting on local distinctiveness we further estimated species richness and evenness per location, based on WPUE. All variables had a variance inflation factor of < 2, indicating a lack of multicollinearity between predictors.

To determine how the local functional distinctiveness of round goby was affected by the selected environmental and biotic variables at each sampling event, we applied a multi-model approach using both Generalised Additive Mixed Models (GAMMs) and Random Forests (RFs). This allows for comparison of the derived response curves and variable importance to assess robustness and sensitivity of results to model choice (Lindegren et al. 2020, 2022). For the GAMM, we applied the following model:

$$\text{Round goby } D_{l,t} = a + s(\text{Bottom oxygen}_{l,t}) + s(\text{Bottom salinity}_{l,t}) + s(\text{Bottom temperature}_{l,t}) + s(\text{Depth}_{l,t}) + s(\text{Chlorophyll}_{l,t}) + s(\text{Exposure}_{l,t}) + s(\text{Richness}_{l,t}) + s(\text{Evenness}_{l,t}) + d(\text{Location x Time step}) + e(\text{Gear}) + \epsilon$$

where the response variable D is the distinctiveness for the round goby at each sampling location l at a specific time t. The parameter a is the intercept, s is the thin plate smooth function for each of the covariates and ϵ the error term. To account for the potential effect of repeating measures within the same area, we also included a random effect d for each sampled location at a certain time (i.e. "Location × Time step" in the formula). The inclusion of this random effect in the model served to account for possible variation in distinctiveness between locations due to their different stages of invasion. Finally, e accounts for the random effects of the different gears used during the sampling. The degrees of freedom of the spline smoother function (s) were constrained to three knots ($k = 3$) to allow for non-linearities, but restricting its flexibility on the model fitting. Since D ranges between 0 and 1, the model was fitted with a beta-regression distribution (Ferrari and Cribari-Neto 2004).

Random forest (RF) is a machine-learning tool comprising ensembles of decision trees that rely on bootstrap aggregation (Breiman 2001). It is capable of producing complex non-linear shapes in single and multiple dimensions, while accounting for interaction amongst all predictors. Random subsets of the data are selected to train individual classification trees within the random forest, whilst the final forest prediction is obtained by averaging predictions across all individual trees. The same response and explanatory variables were used as in the GAMM formulation above. Here, we used the final RF to estimate the relative importance of each predictor (based on 1000 individual trees) to compare it with the importance estimated with GAMMs. In addition, we applied RF to visualise the partial response curves of each explanatory variable.

To evaluate the predictive accuracy of the fitted models between methods, we also performed a formal cross-validation analysis by fitting the same model with a randomly sampled subset of the data (75% of the total observations) and predicting round goby distinctiveness with the remaining 25% of observations that were not used to fit

the models. The cross-validation was repeated 100 times, selecting a new random subset of observations in each iteration for model training and testing. Subsequently, we assessed the range of uncertainty of the predictions (i.e. mean squared error) and the range of explained variance for both methods. All statistical analyses were conducted using the R software, version 4.1.0 (R Core Team 2021) and using the package "mgcv" (Wood 2017) and "randomForest" (Liaw and Wiener 2002).

Results

Functional relationship between round goby and natives

Amongst all species, Eurasian perch (*Perca fluviatilis*) was the most abundant taxa in the monitoring data in terms of weight, representing 24% of the total biomass, followed by common roach (*Rutilus rutilus*; 14.8%) and Atlantic cod (*Gadhus morhua*; 8.3%), while round goby represented only 1.04% of the total biomass (Suppl. material 1: table S3). The estimated functional distinctiveness values (*D*) for the regional species pool ranged from 0.33 (*Gymnocephalus cernuus*) to 0.62 (*Nerophis ophidion*). Round goby had a distinctiveness value of 0.49, which is slightly higher than the median *D* of the fish community (0.44; Fig. 2). This makes round goby the ninth most distinct
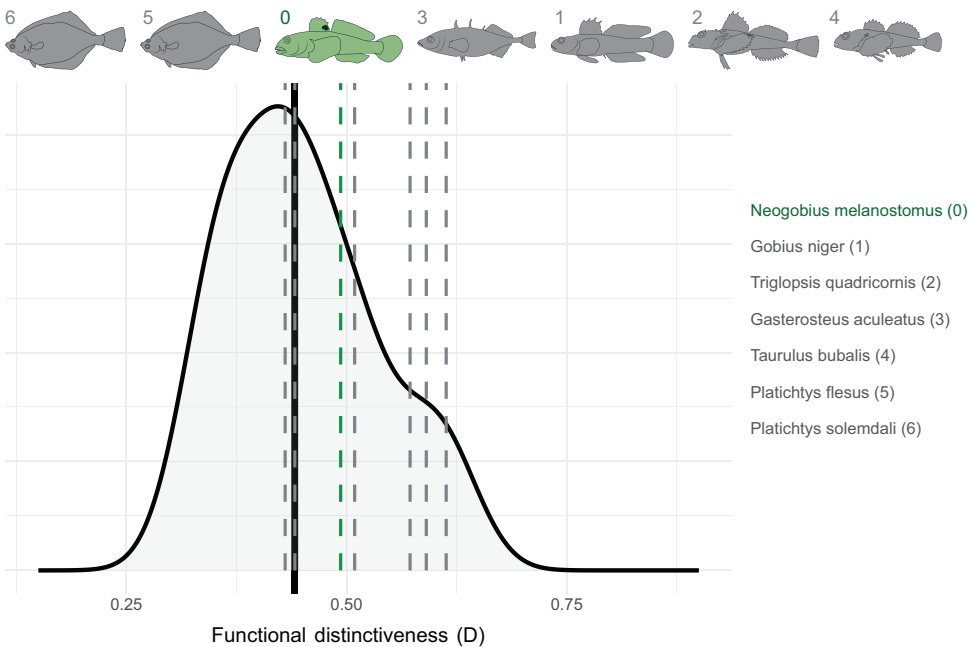
Figure 2. Position of round goby and its six most functionally similar species along the distribution of WPUE-weighted distinctiveness values from the regional species pool. The black vertical line indicates the median value of distinctiveness for the whole community. The highlighted species are ordered according to their values of distinctiveness. The numbers on top of them only indicate the corresponding names.

species out of all 27 species in the dataset. However, round goby was less distinct than four of the six species to which it is most functionally similar (Fig. 2; Suppl. material 1: table S3).

The first two axes of the PCoA of functional distances (trait space) explained 35.1% and 23.2% of the total variability between species, respectively. Round goby was located closer to the most functionally distinct species (defined by the 4th quartile) in the trait space (Fig. 3A). Functionally distinct species were generally defined by displaying some of the following trait modalities: being demersal, strictly benthivorous with rounded fins, having pharyngeal bones, laying eggs in clumps, displaying territorial behaviour and parental care or with no capacity of habitat switching (Fig. 3B, C). In contrast, the most functionally common species (defined by the first quartile) were mostly defined by showing a strictly generalist diet, laying adherent eggs, having forked fins and displaying neither parental care nor showing territorial behaviour (Fig. 3B, C).
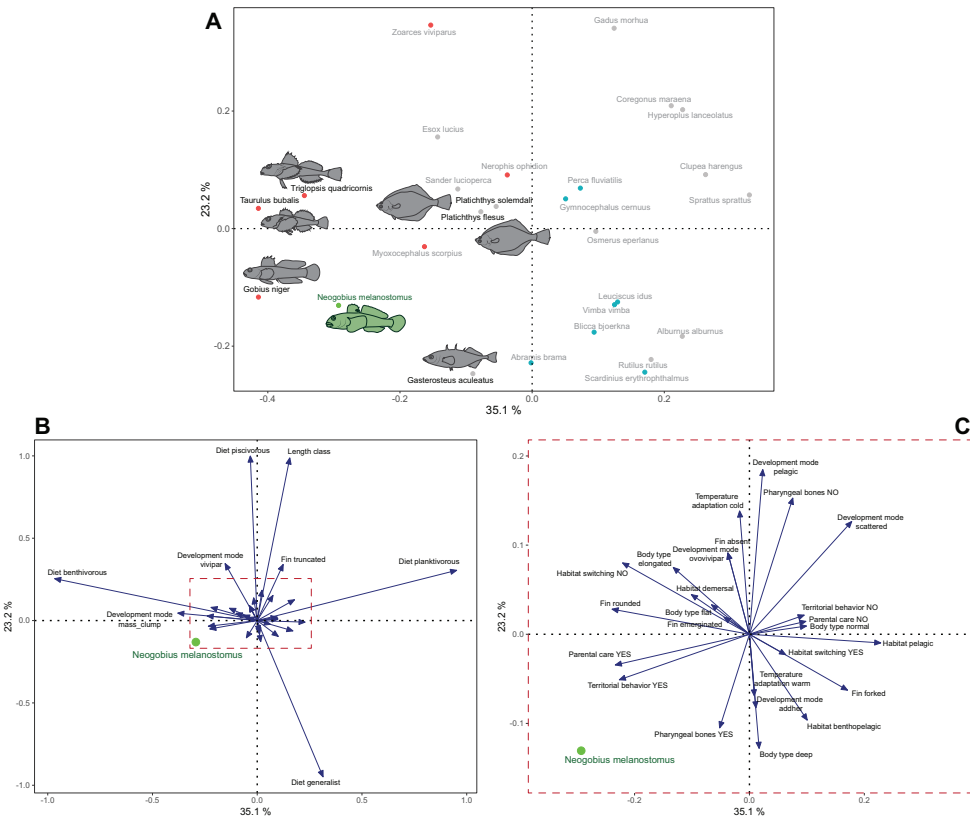
Figure 3. Community trait space given by a PCOA of functional distances between all species (**A**). The green dot and drawing indicate the position of round goby. Red dots indicate the position of species classified as being the most distinct, while blue dots define species classified as most similar compared to the rest of the community. Names in bold indicate the position of the most functionally similar species to round goby **B** biplot of trait vectors and loadings showing which traits are influencing the position of each species in the PCOA **C** zoom of the central part of the biplot.

Key traits affecting round goby distinctiveness

Amongst the set of traits considered, displaying parental care had the highest influence on round goby distinctiveness, with a relative increase of 7.33% in D values when including this trait. This positive effect of parental care was closely followed by having an elongated body type (7.26%), a rounded fin (6.61%) and territorial behaviour (6.40%; Fig. 4). Having a benthivorous diet showed the most negative effect on round goby distinctiveness (-10.28%; Fig. 4), indicating that this trait modality is shared by many species and make round goby less distinct compared to the native community.

The influence of each trait on the whole fish community distinctiveness demonstrated that body shape and fin type had the highest median positive effect (5% and 4.73%), while diet showed the most negative effect (-6.86%; Fig. 4). The positive effects of parental care and territorial behaviour on round goby distinctiveness stand out when compared with the other species, as the median overall effect for these two traits was negative amongst the native species (-6.47% and -5.15%, respectively).
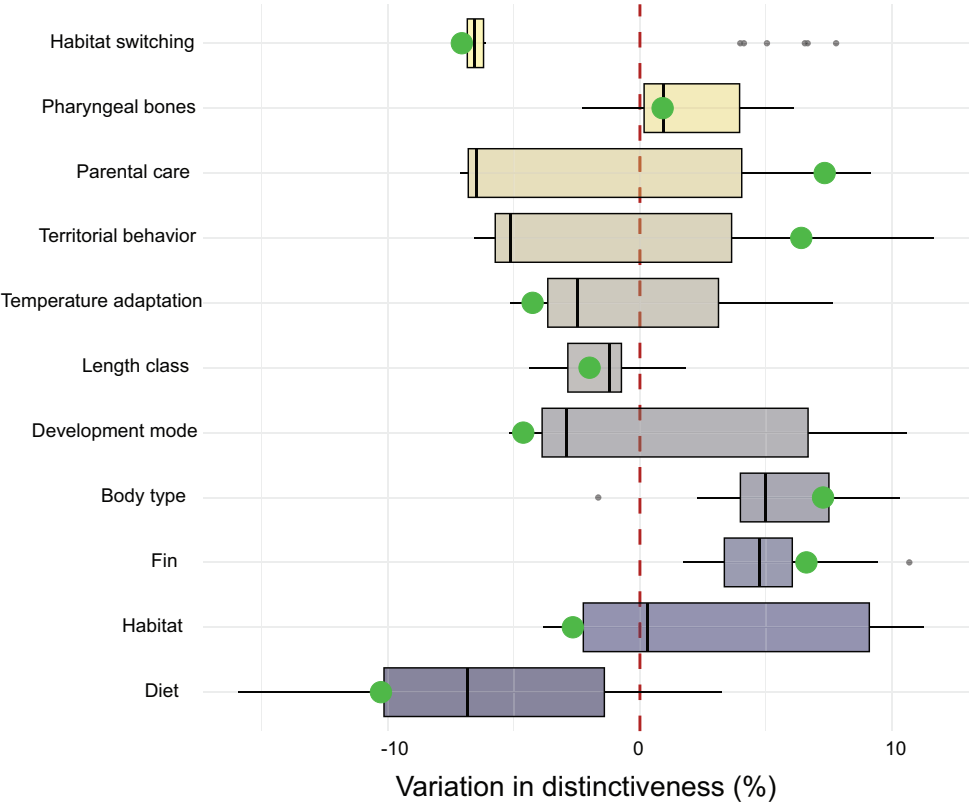
Figure 4. Effect of traits on species functional distinctiveness, shown as the percentage change in overall distinctiveness if excluding each individual trait in the calculations. Results are shown when using either the whole species pool with the green dots representing the effect of each trait on round goby functional distinctiveness.

These values rank round goby as the 2nd species in the regional pool with the highest increase in distinctiveness when including parental care and the 4th when including territorial behaviour.

Spatial patterns and drivers of round goby distinctiveness

Distinctiveness values for round goby at each location were highly variable, with the highest mean value found in Hanöbukten (0.64) and the lowest mean distinctiveness in Herrvik (0.44). In 7 out of 14 locations, the mean distinctiveness of round goby was higher than the overall value when compared to the regional fish community (Fig. 5A). Regarding the change in distinctiveness over time, some variation between and within locations was observed; however, no major changes within locations were detected (Fig. 5B).

From the selected set of potential abiotic and biotic drivers, species richness and evenness, depth, coastal exposure, bottom temperature and oxygen showed significant effects on round goby distinctiveness in the fitted GAMM (Table 2). The relative importance of predictors was similar across across GAMM and RF, showing only slight differences in the order and position of the most important variables (Suppl. material 1: table S4). The partial response curves were also highly similar across methods (Fig. 6). Both species richness and evenness demonstrated a negative non-linear relationship with round goby local distinctiveness (Fig. 6A, B). For species richness, the relationship displayed a U-shaped curve in the GAMM, probably due to the lack of observations from very species rich areas, but a more marked non-linear decrease in the RFs (Fig. 6B). In the case of species evenness, the relationship was dome-shaped, more clearly depicted in the GAMM (Fig. 6A), with the highest distinctiveness observed at evenness values of ~ 0.5. For the abiotic variables, bottom temperature and coastal exposure also showed dome-shaped relationships, with the highest values of round goby distinctiveness found in moderately exposed areas at temperatures ranging from ~ 7.5 to 12.5 °C (Fig. 6D, F). Although the relationship with bottom oxygen was also
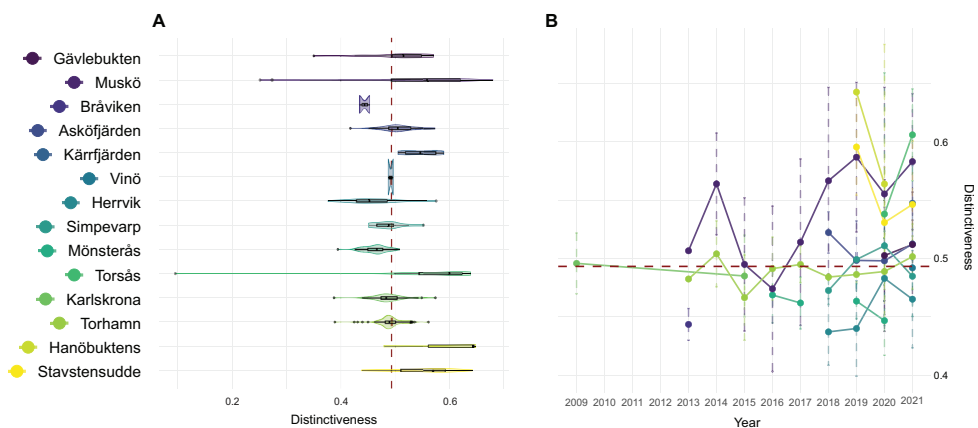

Figure 5. Distribution of round goby local distinctiveness at each sampling site (**A**) and over time (**B**).

Table 2. Results of the GAMM models for round goby local functional distinctiveness. Edf refers to estimated degrees of freedom; significant effects are highlighted in black.

Variables	edf	Chi squared	p-value	R squared	Deviance explained	N	
Eveness	1.803	9.768	**0.015**				*
Richness	1.923	25.361	**< 0.001**				***
Depth	1.001	7.621	**0.006**				**
Bottom temperature	1.906	18.323	**< 0.001**				***
Bottom salinity	1.000	0.370	0.543				
Bottom oxygen	1.000	4.994	**0.025**				*
Chlorophyll	1.004	0.918	0.342				
Exposure	1.927	22.167	**< 0.001**				***
Location x Time step (1st)	1.331	2.314	0.205				
Location x Time step (2nd)	1.812	15.147	**0.017**				*
Location x Time step (3rd)	9.695	198.967	**< 0.001**				***
Gear	0.889	93.769	**< 0.001**				***
				0.498	51.7%	762	
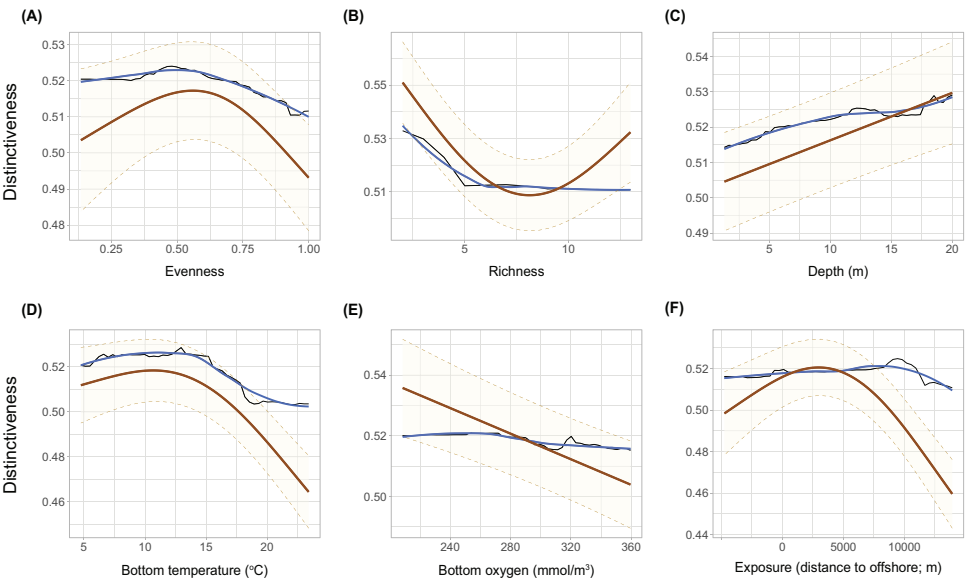
Figure 6. Partial effect curves derived from the models fitted with both GAMM and Random Forest. Only the variables that had a significant effect in GAMMs are shown. The yellow line and ribbon represent the partial curve and the standard deviation derived from the GAMM. The blue and black lines represent the partial effect curve and the corresponding variability derived from the Random Forest.

negative, the trend was linear in the GAMM and dome-shaped for RF, with the highest local distinctiveness values around ~ 220 to 260 mmol/m³ (Fig. 6E). Conversely, depth showed a positive linear relationship with round goby's local distinctiveness in the GAMM and a positive non-linear relationship in RF (Fig. 6C).

Finally, both methods showed similar values of explained variance (i.e. 51.7% for GAMM and 53.8% for RF). The cross-validation analysis demonstrated a better

overall performance for RF, illustrated by lower mean squared error of predicted round goby distinctiveness compared to observation not used for model training (Suppl. material 1: fig. S2).

Discussion

The degree to which NIS display similar or dissimilar traits compared to native species of recipient communities is debated largely due to contrasting results from available studies, primarily conducted in terrestrial ecosystems (Cleland 2011; Escoriza and Ruhí 2016; Xu et al. 2022). Consequently, a better understanding of the trait (niche) overlap amongst native species and NIS, as well as the underlying assembly processes that determine their establishment is needed (Gallien et al. 2014; Gallien and Carboni 2017), especially in marine environments. Our trait-based study focusing on round goby, one of the most widespread invasive fish species in the Northern Hemisphere (Kornis et al. 2012; ICES 2022b) demonstrated that this non-native fish is not particularly distinct in terms of its overall trait composition compared to the native species pool of the Baltic Sea coastal fish community. Although occupying a seemingly isolated position in the community trait space, it does share combination of traits with several other ecologically similar native species, such as black goby (*Gobius niger*), longspined bullhead (*Taurulus bubalis*) and fourhorn sculpin (*Triglopsis quadricornis*).

Although round goby is not generally different from the regional pool of native species in terms of its overall trait composition, we observed notable differences in terms of individual trait modalities, primarily by display of territorial behaviour and parental care. This indicates that native species generally display a reproductive strategy that does not involve defending a territory, nor protecting their offspring. More specifically, round goby males display several types of parental care, including egg inspection, ventilation and nest guarding (Kornis et al. 2012). Egg inspection and ventilation are beneficial for egg survival as they can limit the spread of diseases within the nest, prevent accumulation of sediment and increase the flow of oxygenated water over the eggs (Jones and Reynolds 1999; Meunier et al. 2009). Males can show different types of aggressive behaviour when guarding the nest, such as strength displays, attacking intruders or chasing away potential predators (Wickett and Corkum 1998; Meunier et al. 2009). The expression of male parental care is strongly related to territoriality, especially in sequentially polygynous spawners (mating with multiple females), like round goby (Ah-King et al. 2005). No offspring need to be present to spark aggressive behaviour, as the males can act in a similar way when they defend their territory or shelter from other fishes (Dubs and Corkum 1996; Balshine et al. 2005). Displaying aggression can provide an adaptive advantages, for example, in the protection of offspring or competition for resources, potentially increasing the invasion success of NIS (Chapple et al. 2012). In terms of offspring protection, less aggressive fishes could be easily expelled in a nest intrusion situation (Dubs and Corkum 1996; Balshine et al. 2005). Aggressive territorial defence could also help round goby in securing and protecting good feeding

grounds (Karlson et al. 2007) or even deter potential predators, as being aggressive has been recognised as anti-predator behaviour (Huntingford 1976; Hess et al. 2016). Thus, it is possible that the display of territorial behaviour and parental care by round goby could partly explain its invasion success in the Baltic Sea (Christensen et al. 2021; Puntila-Dodd et al. 2021; Backström and Winkelmann 2022; Behrens et al. 2022). Our findings support the idea that NIS might be successfully established by only differing from natives in terms of one or a few traits, allowing them to cope with existing environmental conditions while, at the same time, colonise more specific or partly vacant niches (Cleland 2011; Gallien et al. 2014; Cadotte et al. 2018; El-Barougy et al. 2020).

While generally similar to native species at the regional scale, our study demonstrates pronounced spatio-temporal variation in terms of local distinctiveness of round goby between and within sampling locations over time. The wide range of values (i.e. from < 0.2 to > 0.6) indicates that round goby can locally co-exist with native species that are either functionally similar or different to itself, reflecting its broad environmental tolerance (Behrens et al. 2017, 2022; Christensen et al. 2021). In terms of the environmental drivers potentially explaining the variation in local distinctiveness, both our methods indicate that round goby appears to be more distinct in colder and deeper monitoring locations with low oxygen and an intermediate level of exposure. These areas are typically inhabited during the winter months following a seasonal offshore-onshore migration (Behrens et al. 2022). The colder, offshore areas in the Baltic Sea are primarily dominated by more marine species, such as Atlantic cod, herring (*Clupea harengus*), sprat (*Sprattus sprattus*) or eelpout (*Zoarces viviparus*) (Olsson et al. 2012; HELCOM 2018; Olsson 2019). These species are generally dissimilar compared to round goby, as they are located almost in an opposite position in the community trait space. In contrast, round goby is functionally more similar to native species in the warmer, shallow and less exposed monitoring locations that are mainly occupied by, for instance, European perch, several species of cyprinids, sticklebacks (Gasterosteidae) and other gobies (Gobiidae) (Olsson et al. 2012; HELCOM 2018). Notably, the three-spined stickleback (*Gasterosteus aculeatus*) and black goby (*Gobius niger*) are two of the six most functionally similar species to round goby, with black goby having fairly similar ecology and habitat requirements (Matern et al. 2021).

The ability to colonise a broad range of habitats and therefore co-exist with different pools of native species with different trait composition may help explain the derived relationships with the biotic variables included in our statistical analysis. For instance, the negative effect of species richness likely reflects the higher local distinctiveness of round goby when co-occurring with the fewer and functionally more dissimilar marine species from colder and deeper locations. Contrarily, when found together with the more species from the native community at more shallow and warmer locations, the likelihood of round goby co-occurring with more functionally similar species is higher, thus explaining its lower local level of distinctiveness at higher richness. This is likely facilitated also by the strong relationship between species and functional richness in the Baltic Sea region (Törnroos et al. 2015; Pecuchet et al. 2016). In terms of evenness, it is assumed that most ecological niches are occupied when species abundances are evenly distributed (Hillebrand et al. 2008). Conversely, highly uneven communities tend to be dominated by the best per-

formers under local environmental conditions that can outcompete functionally-similar species (Hillebrand et al. 2008). The highest local distinctiveness of round goby at low to moderate evenness may indicate a situation where round goby needs to both display similar traits to be able to adapt to the local environment (i.e. environmental filtering), but also being dissimilar (i.e. in this case, territoriality and parental care) relative to the most dominant native species in order to avoid competitive exclusion (Gallien et al. 2014).

In summary, the application of this trait-based approach to the case of round goby in the Baltic Sea shows a partial (trait) niche overlap with native fish species that appears to increase locally when round goby occurs with communities from shallow, inshore and warmer areas. Despite this partial overlap with native species, we also demonstrated that round goby shows pronounced differences compared to the native community in its display of parental care and territorial behaviour. Such differences could play an important underlying role behind round goby's invasion success in the Baltic, as well as in defining the type of interactions with native species. Based on our results, non-aggressive native species that partially share their niche with round goby might be harmed or displaced in the case of direct competition with this NIS for similar resources (e.g. feeding grounds, sheltered areas, nesting sites). Due to the context dependence (i.e. the species and traits selected) of this study, caution should be taken when expanding our conclusions to different scenarios of round goby invasion. For that reason, we encourage the use of similar trait-based approaches, based on functional distinctiveness to further address the invasion of round goby in other areas, with a different environment and species composition (e.g. the North-American Great Lakes or central European rivers). If similar patterns emerge, this would contribute to the understanding of why this species has managed to successfully establish in such different regions, as well as a better understanding if round goby shows similar interactions with native fishes in other areas. Additionally, investigating how round goby dominance could be affected when it co-exists with more similar or dissimilar native species in local communities could also be valuable to define the niche or conditions that this species needs to become invasive (Blackburn et al. 2011). Such an approach could potentially be used to assess the sensitivity of particular areas to the invasion and address potential impacts of round goby on other native fishes (e.g. displacement, competition, facilitation), by combining its environmental preferences with the type of community where round goby appears to be more dominant. Our results and their potential applications may, therefore, be highly relevant if integrated within existing risk assessment tools for biological invasions (Lodge et al. 2016) in order to prioritise and enhance management and conservation actions towards the round goby.

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Supplementary material 1

Supplementary information

Authors: Antoni Vivó-Pons, Isa Wallin-Kihlberg, Jens Olsson, Peter Ljungberg, Jane Behrens, Martin Lindegren

Data type: docx

Explanation note: **table S1.** List of the different modalities for the diet trait. The colored cells indicate the specific modality combination that is displayed by a certain number of species within the regional pool. Numbers inside colored cells indicate the probability that a certain species displays such modality. **table S2.** Trait values for all fish species. The included traits and modalities are further described in Table 1. **table S3.** Functional distinctiveness, WPUE and relative WPUE of the fish species present in the regional pool starting from the most distinct taxon. Quartiles used to classify species distinctiveness are also included. Round goby is highlighted in bold. **table S4.** Importance of predictors for the fitted GAMM and RF. **figure S1.** Correlation between model-derived and in-situ data of bottom temperatureand bottom salinity. **figure S2.** Plots showing the comparison between variance explained and mean squared error (MSE) between GAMM and RF after the cross-validation process.

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RESEARCH ARTICLE

Allelopathy and arbuscular mycorrhizal fungi interactions shape plant invasion outcomes

Xiao Guo[1,2*], Xin-Yue Liu[3*], Si-Yu Jiang[1], Shao-Xia Guo[1], Jing-Feng Wang[3], Yi Hu[3], Shi-Mei Li[1], Hai-Mei Li[1], Tong Wang[1], Ying-Kun Sun[1], Ming-Yan Li[1]

1 *College of Landscape Architecture and Forestry, Qingdao Agricultural University, No. 700 Changcheng Road, Qingdao 266109, China* **2** *Academy of Dongying Efficient Agricultural Technology and Industry on Saline and Alkaline Land in Collaboration with Qingdao Agricultural University, Dongying, 257347, China* **3** *Key Laboratory of Ecological Prewarning, Protection and Restoration of Bohai Sea, Ministry of Natural Resources, School of Life Sciences, Shandong University, 72 Binhai Road, Qingdao, 266237, China*

Corresponding authors: Xiao Guo (xiaoguoyeah@yeah.net); Ming-Yan Li (mingyan413@163.com)

Abstract

The novel weapon hypothesis suggests that allelopathy is an important mechanism for exotic plants to successfully invade native plant communities. Allelochemicals from exotic plants affect both native plants and arbuscular mycorrhizal fungi (AMF) in soil. To explore these effects, we conducted pot experiments using a native plant community comprising of *Chenopodium album* L., *Vitex negundo* L., *Rhus chinensis* Mill., and *Acer truncatum* Bunge. We incorporated AMF strains (*Funneliformis mosseae*, *Glomus versiforme*, and *Rhizophagus intraradices* in a 1:1:1 volume ratio) into a soil mixture comprising equal volumes of autoclaved field soil and grass ash at five concentrations (0 g L^{-1}, CK; 10 g L^{-1}, LRE; 20 g L^{-1}, NRE; 30 g L^{-1}, MRE; and 40 g L^{-1}, HRE) to investigate the allelopathy of *Rhus typhina* L. root and its interactions with AMF on the native plant community. Our results indicated that low concentrations of allelochemicals promoted the relative growth rate and leaf nitrogen content in the native plant community, which was attributed to the increased environmental stress that improved the degree of leaf photosynthetic capacity and organic synthesis rate in the native communities. Moreover, allelochemicals with toxicity decreased the colonization rates of AMF. Meanwhile, the presence of allelopathic effects induced a decrease in leaf nitrogen, and allelopathy altered the effects of AMF on the native community. Specifically, the high concentration of allelochemicals altered the positive effect of AMF on the total aboveground biomass of the native plant community to a negative effect, likely by decreasing colonization rates or affecting soil

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* These authors have contributed equally to this work and share first authorship.

physicochemical properties and the composition of the mycorrhizal communities. Consequently, it is necessary to consider the effects of AMF when testing the roles of allelopathy or the novel weapons hypothesis in biological invasions.

Graphical abstract
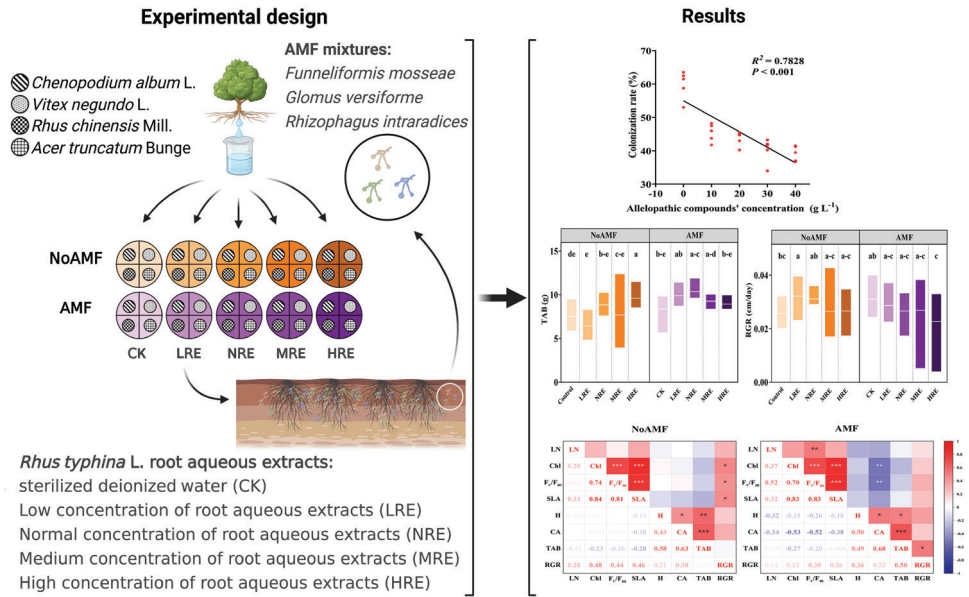
Keywords

Allelochemicals, mycorrhizal symbionts, photosynthetic acquisition capacity, physiological and growth parameters

Introduction

Exotic invaders are a worldwide problem, which can displace native species, causing significant changes in the diversity of native communities (Hassan and Mohamed 2020; Li et al. 2022; Wang et al. 2022a). Exotic plants can successfully establish communities due to their relatively strong allelopathic effects in the invaded habitats (Gruntman et al. 2016). The allelopathy of exotic plants can influence not only the growth and development of native communities but also the composition of mycorrhizal communities (Li et al. 2015; Liu et al. 2017; Hassan and Mohamed 2020). Therefore, comprehending how allelopathy affects native plant communities and mycorrhizal formation can provide valuable insights into invasion mechanisms (Callaway et al. 2011; Gruntman et al. 2016).

Allelopathic effects refer to the positive or negative interaction between plants mediated by chemical compounds (Gross 2003; Cheng and Cheng 2015; Qin et al. 2020). The novel weapons hypothesis posits that non-native species produce secondary chemicals to influence neighboring native species and reduce their competitive advantage (Callaway and Ridenour 2004; Yuan et al. 2021; Yuan et al. 2022). So far, various studies have found inhibitory effects of allelochemicals from exotic plants (Zhang et al. 2011; Hassan and Mohamed 2020; Zhang et al. 2020). For example, invasive plant extracts had negative effects on native seed germination (Yuan et al. 2021). *Centaurea maculosa* inhibits the growth and germination of indigenous species in field soils via the release of the phytotoxin (-)-catechin from its roots (Hierro and Callaway 2003). In addition, invasive plants' allelochemicals can target the mycorrhizal communities, and their outcomes may impact the ecosystem services of native plant communities (Kardol et al. 2006; Gruntman et al. 2016; Bennett and Klironomos 2019).

Arbuscular mycorrhizal fungi (AMF), a representative group of the mycorrhizal communities, can form symbiotic associations with approximately 70% of plant species (Callaway et al. 2011; Weremijewicz et al. 2016; Saia and Jansa 2022). Symbiotic fungi can enhance host plant growth by increasing nutrient absorption and utilization, improving disease resistance, and enhancing drought tolerance (Callaway et al. 2011; Delavaux et al. 2017; de Vries et al. 2020). In return, AMF can aquire carbohydrates derived from host photosynthesis (Sikes et al. 2009; Adomako et al. 2019). Although mycorrhizal symbionts are traditionally considered beneficial to the host (Antunes et al. 2008; Callaway et al. 2011), the association may sometimes have negative consequences (de Vries et al. 2020; Dong et al. 2021). For example, AMF can benefit the host under low phosphorus (P) conditions, whereas the opposite conclusion is drawn under high P conditions (Chen et al. 2020; Dong et al. 2021). Therefore, the effect of AMF on the host may vary depending on environmental conditions.

Invasive plants can produce distinct allelochemicals, which can disturb the structure of mycorrhizal communities in the rhizosphere and alter soil nutrient cycling patterns (Vogelsang and Bever 2009; de Vries et al. 2020). Typically, abundant allelopathic compounds, such as phenolics and flavonoids, are emitted into the surrounding environment through root exudates, volatile organic compounds, leaf leachates, and decomposition of plant materials (Inderjit et al. 2011; Inderjit et al. 2021; Yuan et al. 2022). Root exudates are one of the primary mechanisms to mediate these effects (Hassan and Mohamed 2020). They are released from the living root systems of invasive plants, directly or indirectly affecting the growth and production of native communities (Bennett and Klironomos 2019; Zhang et al. 2020; Dong et al. 2021). Specifically, root exudates from certain invasive species may harm some components of soil biota (Antunes et al. 2008; Vogelsang and Bever 2009). Previous research has demonstrated that invasive and native hosts differ considerably in their preferences for and reliance on AMF (Hawkes et al. 2006; Zhang et al. 2017; de Vries et al. 2020). The root exudates of invasive plants can selectively attract the most beneficial fungal species to facilitate their own growth (Vogelsang and Bever 2009; Zhang et al. 2019).

Consequently, these root exudates may modify the composition of soil microbes. The alterations in mycorrhizal communities, which regulate nutrient availability in soil, may detrimentally affect the growth of native plants, potentially disrupting the symbiotic relationship between AMF and the native communities, thus impeding their access to resources and nutrients (Bennett and Klironomos 2019; Zhang et al. 2019). However, the majority of pertinent research has solely concentrated on the allelopathic impacts of invasive species (Zhang et al. 2011; Hassan and Mohamed 2020; Zhang et al. 2020), with limited emphasis placed on the effects of AMF when assessing the allelopathic effects of biological invasions (Antunes et al. 2008).

In this study, we selected four common plants of the warm temperate zone of China, namely *Chenopodium album* L., *Vitex negundo* L., *Rhus chinensis* Mill., and *Acer truncatum* Bunge, to simulate a native plant community. We used root aqueous extracts of *Rhus typhina* L. to simulate allelopathic effects of invasive plants, and inoculated AMF (*Funneliformis mosseae*, *Glomus versiforme*, *Rhizophagus intraradices*). We conducted a community-level greenhouse experiment to test the following hypotheses: 1) the allelopathic effects of *R. typhina* could negatively impact the growth of the native plant community, 2) the allelopathic effects of *R. typhina* could negatively impact the colonization rates of AMF, and 3) the allelopathic effects of *R. typhina* could alter the impact of AMF on the growth of the native plant community.

Materials and methods

Study species

Rhus typhina L., an exotic tree species indigenous to North America, was introduced to China as a horticultural and greening ornamental plant in 1959 (Hu et al. 2022). Due to its high ecological risk and potent reproductive capacity, it is now considered as one of the most destructive invasive tree species in China (Hu et al. 2022; Xu et al. 2023). To construct a native plant community, we selected four plant species from the warm temperate zone of China that commonly co-occur with *R. typhina*, including one herbaceous species (*Chenopodium album* L.), one shrub (*Vitex negundo* L.), and two trees (*Rhus chinensis* Mill. and *Acer truncatum* Bunge). Given the challenges of cultivating herbaceous plants with consistent growth and development, we dug up *C. album* seedlings with similar growth time directly after the other three plants (*V. negundo*, *R. chinensis*, and *A. truncatum*) had completed their seedling cultivation. Seeds of *V. negundo*, *R. chinensis*, and *A. truncatum* were collected from Mashan, Jimo, Qingdao, Shandong, China in the winter of 2020. We classified each species as invasive or native according to Alien Invasive Flora of China (Ma 2020) and the database Flora of China (www.efloras.org).

In mid-April 2021, we prepared sufficient quantities of seeds of the three selected plant species. The following treatments were performed to improve their germination rate. First, seeds of *R. chinensis* were treated with a baking soda solution at 70 °C for 10

minutes to remove their waxy skins and subsequently cleaning with sterile deionized water. Second, seeds of *A. truncatum* were subjected to a 4 °C treatment for 24 hours. Third, seeds of both *R. chinensis* and *A. truncatum*, as well as those of *V. negundo*, were soaked in fresh water for 24 hours, replacing the water every 12 hours. Soaked seeds were then evenly spread into trays with three layers of gauze on the bottom, and two layers of gauze were placed over the seed surface to improve germination. During this process, sterile deionized water was sprayed into the trays daily to maintain moisture levels. After approximately 30% of the seeds had germinated, we selected strong and uniform seedlings of each species and transferred them to cell trays for further culture. Finally, seedlings of *C. album* with 4–5 leaves were collected from the campus of Qingdao Agricultural University, Shandong, China, until three plant seedlings had developed 4–5 leaves.

Preparation of allelopathic solutions of *R. typhina*

There are two primary rationales for using the root aqueous extracts from *R. typhina* to study the allelopathic effects: (1) allelopathic effects of *R. typhina* are mainly mediated by phenolic substances, most of which, especially polyphenols, can be dissolved in water (Djurdjević et al. 2012; Xu et al. 2023), and (2) in nature, the root exudation pathway is one of the primary methods by which plants release allelochemicals (Hassan and Mohamed 2020; Inderjit et al. 2021). Fully mature *R. typhina* roots were randomly selected from Qingdao Agricultural University, Shandong, China, and gently washed before being cut into small fragments. We added 1000 ml of sterilized deionized water per 100 g of fresh roots at approximately 25 °C for 48 hours, stirring every six hours. The root residues were filtered out to obtain 100 g L^{-1} mother liquor of the *R. typhina* root aqueous extract. Sterilized deionized water was then added in various ratios to the mother liquor to achieve different concentrations of the root aqueous extract (10, 20, 30, and 40 g L^{-1}). The root extract concentration of 20 g L^{-1} represented the normal condition with *R. typhina*'s allelopathic effects (hereafter referred to as NRE) (Xu et al. 2023). Since the concentration of allelochemicals in the soil during the initial period of invasion was low (Zhang et al. 2020), 10 g L^{-1} was designated as the low concentration of the root extract treatment (hereafter referred to as LRE). During the establishment of *R. typhina*, allelochemicals in the soil gradually accumulated (Zhang et al. 2020), so 30 and 40 g L^{-1} were classified as medium and high concentration of the root extract treatments (hereafter referred to as MRE and HRE, respectively). The *R. typhina* root aqueous extracts were refrigerated at approximately 4 °C (for no more than one week), while sterilized deionized water served as the control (0 g L^{-1}; simulating conditions without allelopathic effects; hereafter referred to as CK).

Preparation of AMF

The AMF inoculum comprised a blend of three common AMF strains – *Funneliformis mosseae*, *Glomus versiforme*, and *Rhizophagus intraradices* – in a 1:1:1 volume ratio.

As different AMF types have varying colonization rates in diverse plant species, the AMF mixtures can ensure consistent colonization rates across diverse plant species. In the AMF treatments, each pot was inoculated with the AMF mixtures containing approximately 8×10^3 viable propagules. In the NoAMF treatments, an equivalent amount of sterilized AMF inoculum was added to prevent substrate effects.

Experimental design

The experiment was performed at Qingdao Agricultural University, situated in Qingdao, China (36°31'N, 120°39'E). The region has a temperate monsoon climate with an annual temperature of approximately 12.7 °C, mean annual precipitation of around 821.8 mm, and average yearly sunshine duration of about 2,541.1 h. In the summer of 2021, we conducted a 70-day experiment in an artificially-controlled greenhouse maintained at an average temperature of 29.4 °C and a relative humidity of 77%, using a digital display humidity thermometer, DL-WS20 (Hangzhou Gsome Technology Co., China). The plastic pots used in this study were 20.0 cm in height and 20.0 cm in diameter, and were filled with soil materials consisting of autoclaved field soil and grass ash mixed at a 1:1 volume ratio.

In each plastic pot, we planted four different species of seedlings (*C. album*, *V. negundo*, *R. chinensis*, and *A. truncatum*), with four to five leaves per seedling. One seedling for each plant species was transplanted into one of the four equal sections of the cross lines past the center of the circle within each pot, resulting in a total of four plants per pot. The control treatment consisted of no AMF and root aqueous extracts of *R. typhina*. Overall, we established 100 experimental pots, including two AMF treatments (with and without AMF) and five concentrations (0 g L^{-1}, CK; 10 g L^{-1}, LRE; 20 g L^{-1}, NRE; 30 g L^{-1}, MRE; and 40 g L^{-1}, HRE). Each treatment had ten replicates ($n = 10$), and all individuals of each species and treatments were randomly distributed. Over the entire experiment, we added 50 ml of the appropriate concentration solution of the root aqueous extracts from *R. typhina* once every two days in the five concentration treatments, for a total of thirty times. Weeding and pest control measures were implemented as usual and the greenhouse was adequately ventilated. All pots were placed randomly within the greenhouse and rotated normally to minimize any potential effects of environmental differences during the experiment.

Harvest and measurements

Physiological parameters measured in the native plant community included specific leaf area (*SLA*), total chlorophyll concentration (*Chl*), F_v/F_m, and leaf nitrogen (*LN*), which can characterize the degree of leaf photosynthetic capacity (Wang et al. 2020). Growth parameters included plant height, crown area (*CA*), relative growth rate (*RGR*), and total aboveground biomass (*TAB*). Plant height and *CA* characterize the ability of the native plant community to acquire light (Wang et al. 2020; Wang et al. 2022b). *RGR* describes the rate of plant growth. At the conclusion of the experiment, the

intertwining of root systems in each plastic pot prevented the measurement of belowground biomass for each plant. Consequently, we measured the total aboveground biomass (*TAB*) of all four plants to represent the community's growth. Additionally, the AMF colonization rate was used to characterize the quantity of mycorrhizal symbiont formation under different conditions.

Prior to the treatment, the aboveground component of five seedlings from each indigenous species with 4–5 true leaves were oven-dried at 105 °C for 0.5 h to inactivate them. The drying process was continued for an additional 48 h at 80 °C, and then the weight was measured. The mean value was regarded as the initial biomass for each species.

After 60 days, when the herbaceous flora had reached the reproductive stage, the maximum growth index of all native plants was determined. The vertical height of the plant from the base to the terminal bud was gauged as the plant height. The *CA* was calculated using the diamond-shaped area formula (Wang et al. 2022b; Guo et al. 2023b).

$$CA = 0.5 \times a \times b$$

where *a* indicates the length of the plant's maximum horizontal extension, and *b* signifies the length perpendicular to *a* on the plant's maximum horizontal extension plane.

For each plant, chlorophyll fluorescence parameters were evaluated during sunny and cloudless weather conditions from 8:30 to 11:30 using Pocket PEA (Hansatech Instruments Ltd., UK). The top-to-bottom first fully developed leaf was selected, and after undergoing dark treatment for 0.5 h, the maximum fluorescence value (F_m) and variable fluorescence value (F_v) were evaluated. The maximum photochemical efficiency (F_v/F_m) of PSII was then calculated (Hu et al. 2022; Xing et al. 2022).

The second or third fully expanded and healthy leaf from the apical meristem of each plant was selected, cleaned with sterilized deionized water, and the leaf area was measured using a portable leaf area meter (Yaxin-1241, Yaxin Inc., Beijing, China). The leaves were inactivated to inactivation at 105 °C for 0.5 h and dried at 85 °C for 24 h in a drying oven until they reached constant weight to obtain their dry weights. The *SLA* was calculated using the formula (Wang et al. 2022b; Guo et al. 2023a).

$$SLA = leaf\ area \div dry\ weight$$

The total chlorophyll content was determined using the ethanol extraction method. Four fully mature leaves near the top of each plant (one leaf per plant) were collected for each treatment and chopped to approximately 0.2 g fresh weight. The leaves were then submerged in 10 ml of 95% alcohol until completely whitened. The absorbance of chlorophyll in the solution at 665 nm and 649 nm wavelengths was measured using a UH5300 UV/VIS spectrophotometer (Hitachi, Inc., Tokyo, Japan). The concentrations of chlorophyll *a*, *b*, and total chlorophyll were calculated using the following formulas (Wang et al. 2022b; Guo et al. 2023a):

$$Chlorophyll\ a\ concentration = 13.95 \times A_{665} - 6.88 \times A_{649}$$

$$Chlorophyll\ b\ concentration = 24.96 \times A_{649} - 7.32 \times A_{665}$$

$$Chlorophyll\ content$$
$$= Chlorophyll\ concentration$$
$$\times volume\ of\ extraction\ solution\ /\ fresh\ weight\ of\ sample$$

$$Chl = Chlorophyll\ a\ content + Chlorophyll\ b\ content$$

The aboveground parts of the plants were harvested, washed, classified according to species, inactivated at 105 °C for 0.5 h in the oven, and dried in the oven at 80 °C for 48 h. The total aboveground biomass of all species in the same pot was measured and the *RGR* of native plants was calculated using the formula (Wang et al. 2022b).

$$RGR = (ln\ X_1 - ln\ X_2) \div \Delta T$$

where X_2 and X_1 denote the final and initial biomass, respectively, and ΔT is the duration of the experiment.

For *LN* measurements, dried leaves of each species were ground and weighed to 0.5 g using the Kai-style digestion method (K9860, Hanon, Shandong, China).

Roots were collected from 70-day plants. Fine roots (15 g) were excised from each plant, washed, and stained with acid fuchsin to determine the percentage of mycorrhizal colonization by the method of Biermann and Linderman using a BX50 Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan) (Biermann and Linderman 1981).

Data analyses

The Community-Weighted Trait (*CWT*) method was utilized to evaluate the relevant physiological parameters (leaf nitrogen, total chlorophyll concentration, F_v/F_m, and specific leaf area) and growth parameters (height, crown area, and relative growth rate) of the native plant community in this study (Wang et al. 2020). *CWT* was calculated according to the following formula:

$$CWT = \sum_{i=1}^{S} P_i \times X_i$$

where *S* is the total number of species and P_i represents the relative abundance of species i in the native community. $P_i = n_i \div N$, n_i and N represent the plant number of species *i* and the number of plants of all species in the native plant community, separately. X_i denotes the average value of morphological and physiological characteristics of species i.

To assess differences in growth and physiological traits among different treatments, we conducted a two-way analysis of variance (*ANOVA*). Prior to the *ANOVA*, we performed variance homogeneity tests for each group of data and transformed unevenly

distributed data. We used Duncan's test with a significance level of $\alpha \leq 0.05$. For each concentration of allelochemicals, we performed linear regressions to determine the relationship between AMF colonization rates and the concentration of root aqueous extracts from *R. typhina*. To evaluate the correlation level of the main traits for the native plant community, we generated Pearson product-moment correlation coefficients among the physiological and growth parameters. We used the IBM SPSS Statistics 25.0 software package (IBM Corporation, Armonk, NY, USA) to conduct all analyses. We created all figures using Origin 2021 (OriginLab Co., Northampton, MA, USA).

To gain an understanding of how growth and physiological parameters of the native plant community were influenced by allelopathy and AMF, and how these parameters were moderated, structural equation modeling (*SEM*) was utilized. A conceptual model, based on theoretical interactions among variables, was established to serve as our formal hypothesis. To test our hypotheses statistically, the variance-covariance matrix implied by the conceptual model was compared with the observed variance-covariance matrix, and maximum likelihood estimation was employed to estimate model coefficients. Model fit was considered acceptable if the χ^2 test was non-significant, the root mean square error of approximation (*RMSEA*) was low (*RMSEA* < 0.01), and the comparative fit index (*CFI*) was high (*CFI* > 0.90). *SEM* was conducted using the *lavaan* and *semPlot* package in R 4.2.3 (R Core Team 2023).

Results

Plant physiology

Physiological parameters such as *LN* and *Chl* were significantly affected by allelopathy ($p < 0.001$, Table 1). In detail, *LN* values were improved by the NRE and MRE treatments, increasing by 32% and 28% respectively compared to the control (Table 1, Fig. 2A), whereas *Chl* was higher in the NRE treatment than the LRE, MRE, and HRE treatments ($p = 0.037$, Table 1, Fig. 2B), which resulted in a 34%, 46%, and 57% advance respectively. Furthermore, the allelopathic effects had negligible effect on F_v/F_m and *SLA* (Table 1, Fig. 2C, D).

The AMF inoculation significantly affected only *LN*, which was depressed within the LRE, NRE, MRE, and HRE treatments ($p < 0.001$, Table 1, Fig. 1A), leading to a 15%, 35%, 28%, and 29% reduction compared to the corresponding NoAMF treatments respectively. According to the results of the two-way ANOVA, inoculating AMF did not significantly affect *Chl* (Table 1), while multiple comparisons showed that *Chl* was reduced by 31% in the NRE treatment compared to the corresponding NoAMF treatments (Fig. 2B). Additionally, all other physiological parameters, including *SLA* and F_v/F_m, were not impacted by AMF (Table 1, Fig. 2C, D).

Most physiological trait values were not influenced by the interaction between allelopathic effects and AMF treatments, such as *Chl*, F_v/F_m, and *SLA* (Table 1, Fig.2B–D). Nevertheless, the interaction between allelopathic effects and AMF had a significant

Table 1. Results of two-way *ANOVA* for the effects of allelopathy, AMF and their interaction on growth and physiological parameters of native plant community. *CA*, crown area; *RGR*, relative growth rate; *TAB*, total aboveground biomass; *LN*, leaf nitrogen; *Chl*, total chlorophyll concentration; *SLA*, specific leaf area. Data are presented with *F* value (*n* = 10). Significant effects are indicated by bold font ($p \leq 0.05$).

	Growth parameters				Physiological parameters			
	CA	Height	*RGR*	*TAB*	*LN*	*Chl*	F_v/F_m	*SLA*
Allelopathy	1.274	2.109	**2.567**	2.183	**34.661**	**2.671**	0.257	1.208
AMF	1.011	2.259	1.041	**3.589**	**241.722**	1.921	3.662	0.004
Allelopathy × AMF	0.283	2.288	2.254	**4.716**	**24.352**	1.667	1.172	0.702
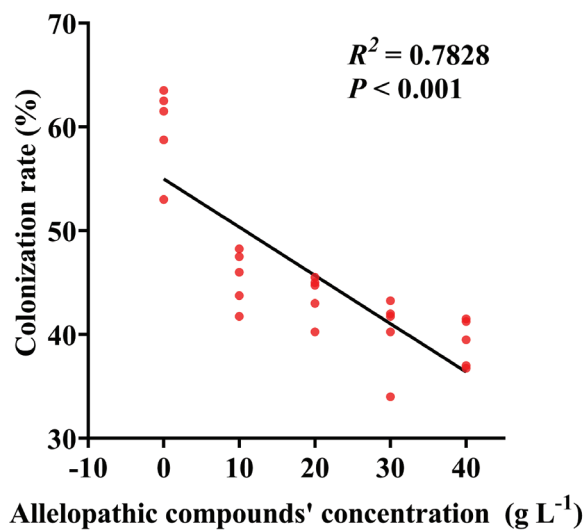
Figure 1. Responses of AMF colonization rate to the different concentrations of allelochemicals (*n* = 5). Five different concentrations of *Rhus typhina* L. root aqueous extracts were used, which were 0, 10, 20, 30, and 40 g L^{-1}, respectively. The proportion of variance is explained by R^2. The regression line was significant at the $p < 0.05$ level.

effect on *LN* of the native plant community (Table 1). The LRE, NRE, and HRE treatments decreased *LN* by 13%, 14%, and 30% respectively, compared to the control ($p < 0.001$, Fig. 2A).

Plant growth

The two-way ANOVA indicated that only *RGR* was altered by allelopathic effects ($p = 0.043$, Table 1). Specifically, the LRE treatments raised *RGR* by 25% compared to the control (Table 1, Fig. 3D). The allelopathic effects had no significant effects on plant height, *CA*, and *TAB* (Table 1, Fig. 3A–C). However, according to the multiple comparisons, *TAB* was significantly improved by the HRE treatment, increasing 40% by compared to the control (Table 1, Fig. 3C). Further analysis of four individual species
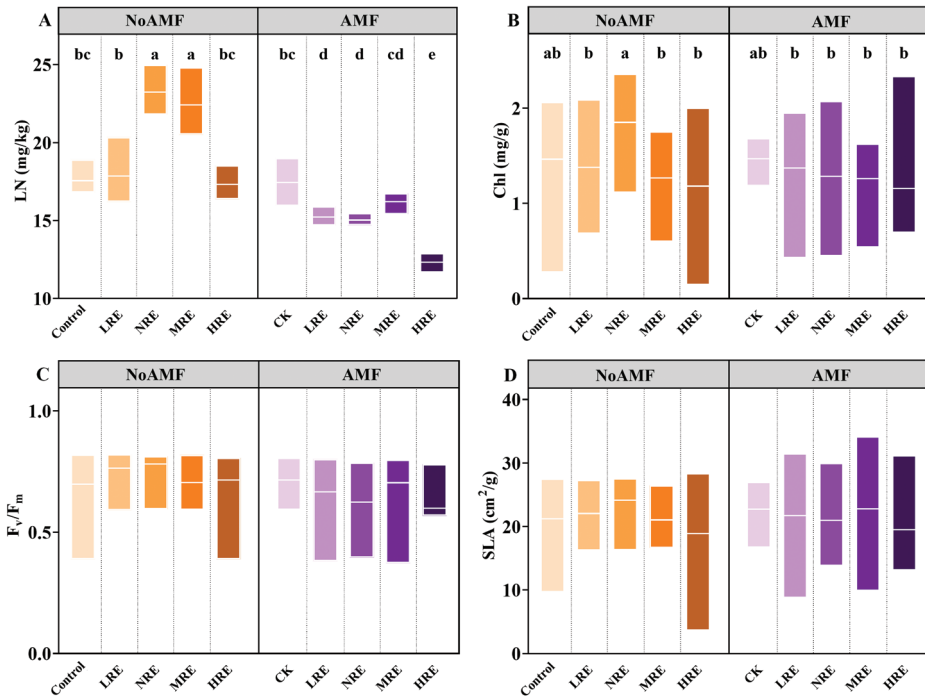
Figure 2. Responses of physiological parameters of native plant community to the allelopathy and its interactions with AMF (n = 10). The boxes represent the range of maximum and minimum values, with a bold horizontal line indicating the average value. Colors indicate different AMF treatments (orange, AMF; purple, NoAMF). The physiological parameters include leaf nitrogen (*LN*) (**A**) total chlorophyll concentration (*Chl*) (**B**) F_v/F_m (**C**) and specific leaf area (*SLA*) (**D**). There were five different concentrations of the root aqueous extracts of *Rhus typhina* including 0 g L^{-1} (CK), 10 g L^{-1} (LRE), 20 g L^{-1} (NRE), 30 g L^{-1} (MRE), and 40 g L^{-1} (HRE). The different letters indicate a significant difference ($p \leq 0.05$) with Duncan's test.

found that the HRE treatment significantly improved the aboveground biomass of only *C. album* (Suppl. material 1: fig. S1A).

Inoculation with AMF increased *TAB* by 57% in the LRE treatment and decreased it by 29% in the HRE treatment compared to the NoAMF treatment (p = 0.006, Table 1, Fig. 3C). Overall, plant height was not influenced by AMF while the LRE treatment increased plant height by 34% compared to the corresponding NoAMF treatments (Table 1, Fig. 3A). Moreover, AMF treatments did not significantly change all other growth parameters, including *CA* and *RGR* (Table 1, Fig. 3B, D).

The interaction between allelopathic effects and AMF significantly affected on *TAB* and the LRE and NRE treatments increased *TAB* by 48% and 45% respectively, compared to the control (p = 0.005, Table 1, Fig. 3C). Meanwhile, the LRE treatment resulted in a 27% advance in plant height compared to the control (Fig. 3A). Nevertheless, the *CA* and *RGR* were not influenced by the interaction between allelopathic effects and AMF treatments (Table 1, Fig. 3B, D).
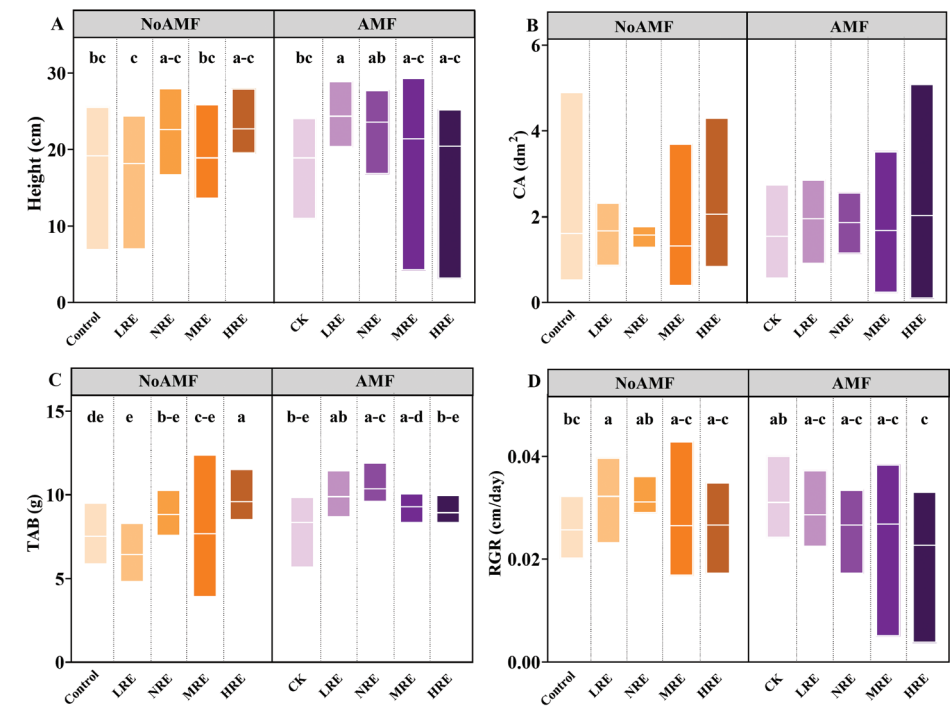
Figure 3. Responses of the growth parameters of native plant community to the allelopathy and its interactions with AMF (n = 10). The boxes represent the range of maximum and minimum values, with a bold horizontal line indicating the average value. Colors indicate different AMF treatments (orange, AMF; purple, without AMF). The growth parameters include height (**A**) crown area (*CA*) (**B**) total aboveground biomass (*TAB*) (**C**) and relative growth rate (*RGR*) (**D**). There were five different concentrations of the root aqueous extracts of *Rhus typhina* including 0 g L^{-1} (CK), 10 g L^{-1} (LRE), 20 g L^{-1} (NRE), 30 g L^{-1} (MRE), and 40 g L^{-1} (HRE). The different letters indicate a significant difference ($p \leq 0.05$) with Duncan's test.

Trait correlation

We examined 28 pairs of growth and physiological traits in the native community using a Pearson correlation test. Nine pairs for the NoAMF treatments and 10 pairs for the AMF treatments were significantly correlated at $p \leq 0.05$ (Fig. 4). The six correlated pairs of the NoAMF treatments were also correlated in the AMF treatments. The different three correlated pairs of the NoAMF treatments were *RGR* and *SLA*, *RGR* and *Chl*, *RGR* and F_v/F_m, which were positively correlated (Fig. 4). The different four correlated pairs of the AMF treatments were *CA* and *Chl*, *CA* and F_v/F_m, which were negatively correlated, while *LN* and F_v/F_m, *RGR* and *TAB* were positively correlated (Fig. 4).

The *SEM*s were well-ftted to the data (χ^2 = 4.507, df = 11, P = 0.953, CFI = 1.000, $RMSEA$ = 0.000). Allelopathy had direct negative associations with relative growth rate, total chlorophyll concentration, specific leaf area, leaf nitrogen and F_v/F_m but positive associations with height, crown area and total aboveground biomass (Fig. 5). AMF had a negative direct association with all plant traits except height and total
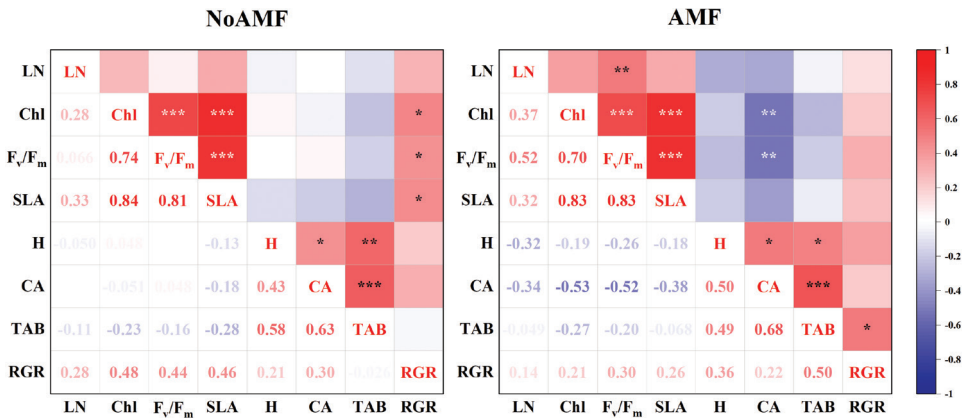
Figure 4. Matrix of Pearson product-moment correlation coefficients for the growth and physiological traits of the native community across different AMF treatments (n = 10). Traits include leaf nitrogen (*LN*), total chlorophyll concentration (*Chl*), F_v/F_m, specific leaf area (*SLA*), plant height (*H*), crown area (*CA*), total aboveground biomass (*TAB*), and relative growth rate (*RGR*). Significant correlations are denoted by bold font and asterisks: *** $P \le 0.001$, ** $P \le 0.01$, and * $P \le 0.05$.

$\chi^2 = 4.507$; df = 11; $P = 0.953$; CFI = 1.000; RMSEA = 0.000
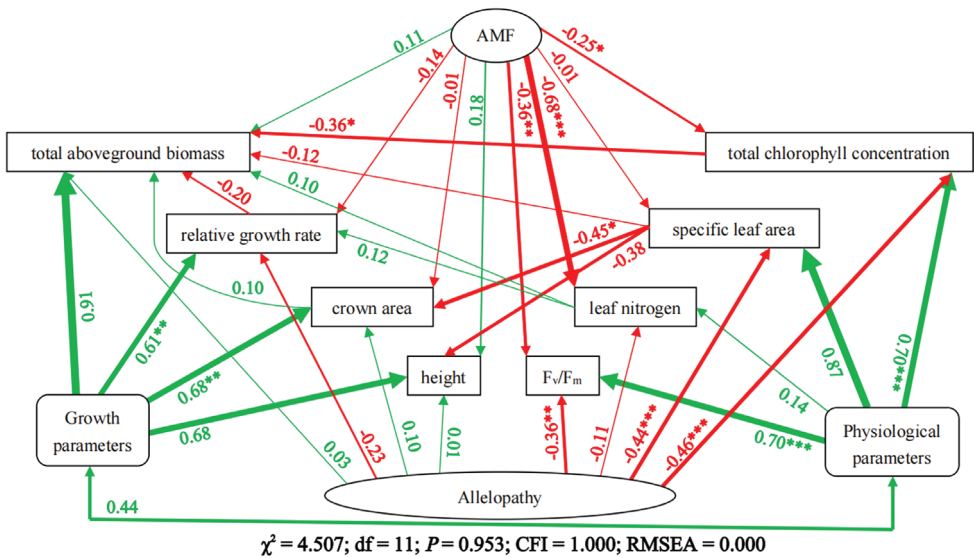
Figure 5. The structural equation models relating the growth and physiological parameters of the native plant community. Green lines indicate positive relationships while red lines indicate negative relationships. Line thickness depicts the magnitude of path coefficients. Significant effects are indicated by asterisks: *** $P \le 0.001$, ** $P \le 0.01$, and * $P \le 0.05$.

aboveground biomass (Fig. 5). Although leaf nitrogen had a positive association with relative growth rate and total aboveground biomass, the association was not statistically significant (p = 0.455; p = 0.470, Fig. 5). Further, AMF had a negative direct association with all plant traits except height and total aboveground biomass (Fig. 5). Total

aboveground biomass was also positively linked to relative growth rate, total chlorophyll concentration and specific leaf area, but negatively related to crown area and leaf nitrogen. Also, growth parameters had a strong and positive relationship with height, crown area, relative growth rate and total aboveground biomass. In turn, physiological parameters had direct positive associations with total chlorophyll concentration, specific leaf area, leaf nitrogen and F_v/F_m (Fig. 5). Finally, growth parameters had a direct association with physiological parameters (Fig. 5).

Mycorrhizal colonization

The colonization rates of mycorrhizal in the native plant community were negatively correlated with the concentrations of root aqueous extracts from *R. typhina* ($p < 0.001$; $R^2 = 0.7828$, Fig. 1). The AMF colonization rates in the LRE, NRE, MRE, and HRE treatments were significantly reduced by 25%, 27%, 33%, and 35% respectively compared to the control (Fig. 1).

Discussion

The allelopathy and its interactions with AMF affected the physiological and growth parameters of the native plant community. In general, the low concentration of allelochemicals promoted *RGR* and *LN* in the native plant community, while the present of allelochemicals altered AMF effects on the native community.

Responses of native plant community to allelopathy

Allelopathy, as a plants' defense mechanism, could alter the physical and chemical properties of the soil conditions (Lorenzo et al. 2013). The normal concentration of allelochemicals can produce stress on the growth of native plants. In response to the stress, the native plant community allocated or produced more *LN* and *Chl* to improve the degree of leaf photosynthetic capacity and organic synthesis rate. This observation was consistent with previous studies that compared the photosynthetic properties of native plant species in plots invaded and non-invaded by alien species (Wang et al. 2020). As reported, the ability of sunlight acquisition and the degree of leaf photosynthetic capacity of native plant species were prominently improved under invasion by *Solidago canadensis* and *Erigeron annuus* (Wang et al. 2020). In our experiments, when the concentration of allelopathic compounds was above a certain threshold value, the stress no longer influenced the native plant community.

Allelochemicals have long been considered as a mechanism by which invasive species eliminate natives (Bais et al. 2003; Gruntman et al. 2016; Hassan and Mohamed 2020). Nevertheless, few studies have found that allelochemicals of invasive plants have neutral or positive effects on native plants (Gross 2003; Zhang et al. 2020). Moreover, it is important to note that the source heterogeneity of allelochemicals can affect the

allelopathic effects (Zhang et al. 2020). In this study, we investigated the allelopathic effects of root aqueous extracts of *R. typhina* on native plant communities. In conclusion, the low concentrations of allelochemicals promoted *RGR*, whereas the medium and normal concentrations of allelochemicals fostered *LN* in native plant communities, contradicting our first hypothesis. In another study, the leaf aqueous extracts of *R. typhina* decreased all seed germination indices and radicle length of the native plant *Lactuca sativa* L. (Xu et al. 2023). Although disturbances from competition among plants were excluded when using the extracts to explore the allelopathic effects of invasive plants, the different simulating ways might make the conclusions inconsistent (Callaway et al. 2008; Zhang et al. 2020). Since the allelopathic effects of *R. typhina* were simulated only by the root aqueous extracts in our study, it is reasonable that our findings differed from those of experiments involving individual plants of *R. typhina* or using other simulations.

Additionally, we found that the total aboveground biomass of the native plant community was significantly higher in the HRE treatment than in the other extract treatments. We analyzed the allelopathic effects on the aboveground biomass of four species (*C. album*, *A. truncatum*, *R. chinensis*, and *V. negundo*) that constituted the native plant community separately. Only the aboveground biomass of *C. album* increased significantly with the HRE treatment, while the aboveground biomass of the remaining three plants remained unchanged statistically. A previous study suggests that under the presence of allelopathic compounds, distinct plant species exhibited varying germination and growth rates (McEwan et al. 2010). Therefore, we speculate that these results might be due to a superior defense of *C. album* against allelochemicals.

Responses of the native plant community to AMF and allelopathy

AMF can facilitate host plants growth and provide them with competitive advantages (Antunes et al. 2008; Delavaux et al. 2017; Adomako et al. 2019). However, our results suggest that AMF induced *LN* decline of the native community under the influence of allelopathic effects of *R. typhina*. The presence of allelopathic effects was considered as an environmental stress. In response to this stress, the degree of leaf photosynthetic capacity of the native plant community was reduced when AMF existed. Furthermore, under allelopathic effects alone on the native plant community, the response of *LN* was inconsistent with the results obtained from the combined effects of allelopathy and AMF. The Pearson product-moment correlation coefficients among the growth and physiological traits indicated that *CA* and F_v/F_m had a negative correlation, while *LN* and F_v/F_m, *CA* and *TAB* positively correlated. In the presence of AMF, low concentrations of allelochemicals hindered *LN* but promoted *TAB* in the native plant community. This suggests that native plants prioritize absorbing a greater amount of resources from the soil to resist environmental stress rather than enhancing the degree of leaf photosynthetic capacity in response to mild allelopathy. Based on the resource optimization hypothesis (Agren and Franklin 2003), we speculate that the host plant allocates more available resources to promote the growth of belowground parts. The

inoculated AMF can combine with the host plant roots to generate mycorrhizal symbionts that can obtain additional water and nutrients from the soil, contributing to the growth and development of the native plant community (Agren and Franklin 2003).

While the significance of AMF alone in soil has long been recognized in relation to plant invasions, few studies have examined the influence of AMF on allelopathic effects in the context of communities (Pellegrino et al. 2015; Bennett and Klironomos 2019). Our results showed that the *TAB* was differently affected by the AMF treatment among the growth parameters treated with different strengths of the allelopathic effects. Specifically, AMF treatments significantly promoted *TAB* of the native plant community in the LRE treatment. Although few empirical experiments have investigated the effects of soil microbes on plant allelopathy, available experiments have found that soil microbes can counteract allelopathy by degrading the secondary metabolites secreted by the plants, which could reduce or completely eliminate allelopathy (Saia and Jansa 2022). This function might have mitigated the negative effects of allelopathy on AMF.

The high concentration of allelochemicals transformed the promotive effect of AMF on *TAB* into the inhibitory effect. Our results suggest that the high concentration of allelochemicals had a neutral impact on the native plant community. Therefore, this phenomenon can be attributed to two plausible reasons: (1) the allelopathic effects from invasive plants can influence patterns of soil nutrient cycling and the soil nutrient environment, altering the effect of AMF on the host (Zhang et al. 2017; de Vries et al. 2020); (2) allelopathy can decline the AMF colonization rates, influencing the mycorrhizal beneficial symbiosis and reducing the uptake and utilization of water and nutrients from the soil by native plants (Zhang et al. 2017; de Vries et al. 2020). Invasive and native hosts have different preferences for AMF and high concentrations of allelochemicals from *R. typhina* with strong toxicity may filter the AMF in the soil and accumulate beneficial fungal species to promote their own growth (Antunes et al. 2008; Inderjit and van der Putten 2010; Inderjit et al. 2021), which could also explain the reduction of AMF colonization rates. The filtering could result in a reduction of mycorrhizal communities helpful for the growth of native plant communities (Antunes et al. 2008).

Conclusion

As biological invasions continue to worsen, it is increasingly necessary to explore the mechanisms behind successful species invasions (Zhang et al. 2020). In this study, we conducted experiments and found that the growth and physiological parameters of the native plant community were affected by allelopathy and its interactions with AMF. Our results indicated that the *RGR* and *LN* of the native plant community were increased by the low concentration of allelochemicals, promoting leaf photosynthetic capacity and organic synthesis rate. Meanwhile, due to allelopathic stress from exotic plants, AMF inhibited *LN* in the native community. The high concentration of allelochemicals turned the positive effect of AMF on the native plant community into a negative effect, which was probably caused by a decrease in the colonization rates

of AMF or by affecting soil physicochemical properties and mycorrhizal community composition. Our findings offer additional theoretical support for managing the invasion of exotic plants in temperate China. For the management and control of invasive plants, we propose planting native plants tolerant to the allelopathic effects of *R. typhina* around invaded areas to establish native communities. This measure may reduce the impact of *R. typhina* on native communities and slow down the rate of invasion. Nevertheless, further experiments are necessary to verify their feasibility.

Acknowledgements

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Supplementary material 1

Responses of the aboveground biomass of native plant species to the allelopathy (n = 10)

Authors: Xiao Guo, Xin-Yue Liu, Si-Yu Jiang, Shao-Xia Guo, Jing-Feng Wang, Yi Hu, Shi-Mei Li, Hai-Mei Li, Tong Wang, Ying-Kun Sun, Ming-Yan Li

Data type: tif

Explanation note: The boxes represent the range of maximum and minimum values, with a bold horizontal line indicating the average value. The plant species include *Chenopodium album* L. (A), *Vitex negundo* L. (B), *Rhus chinensis* Mill. (C), and *Acer truncatum* Bunge (D). There were five different concentrations of the root aqueous extracts of *R. typhina* including 0 g L^{-1} (CK), 10 g^{-1} (LRE), 20 g L^{-1} (NRE), 30 g L^{-1} (MRE), and 40 g L^{-1} (HRE). The asterisk (*) indicates a significant difference ($p \leq 0.05$) with Duncan's test.

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Germany's first Action plan on the pathways of invasive alien species to prevent their unintentional introduction and spread

Katharina Mayer[1], Tina Heger[2,3,4], Ingolf Kühn[5,6,7],
Stefan Nehring[8], Mirijam Gaertner[1,9]

1 *Nürtingen-Geislingen University of Applied Sciences (HFWU), Schelmenwasen 4-8, 72622 Nürtingen, Germany* **2** *Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB), Müggelseedamm 310, 12587 Berlin, Germany* **3** *Freie Universität Berlin, Institute of Biology, Königin-Luise-Str. 1–3, 14195 Berlin, Germany* **4** *Technical University of Munich, TUM School of Life Sciences, Restoration Ecology, Emil-Ramann-Str. 6, 85350 Freising, Germany* **5** *Helmholtz Centre for Environmental Research – UFZ, Dept. Community Ecology, Theodor-Lieser-Str. 4, 06120 Halle, Germany* **6** *Martin Luther University Halle-Wittenberg, Geobotany & Botanical Garden, 06108 Halle, Germany* **7** *German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Puschstraße 4, 04103 Leipzig, Germany* **8** *Federal Agency for Nature Conservation, Konstantinstr. 110, 53179 Bonn, Germany* **9** *Centre for Invasion Biology, Department of Botany and Zoology, Private Bag X1, 7602 Matieland, Stellenbosch University, South Africa*

Corresponding author: Mirijam Gaertner (gaertnem@gmail.com)

Abstract

The increase in global trade and traffic networks contributes to the introduction and spread of invasive alien species, posing a threat to biodiversity. EU Regulation 1143/2014 addresses the prevention and management of invasive alien species and requires an action plan on the priority pathways of unintentional introduction and spread of invasive alien species by each member state. To this end, the first German action plan was developed for the German government in cooperation with relevant Ministries and authorities, scientists, administrative experts, stakeholder working groups and political as well as public deliberation processes. As a result, 14 priority pathways of unintentional introduction, escape or release and spread were identified, resulting in 24 targeted measures. Nineteen sectors will be involved in the implementation of these measures. Here we describe the development process and outcome of Germany's first action plan. By this, we aim making the process public and transparent, which can facilitate a revision of the action plan required at least every six years, and trigger broader European as well as national cooperation in the future.

Introduction

Invasive alien species are a major driver of global biodiversity loss (e.g., Vilà et al. 2011; Bongaarts 2019; Pyšek et al. 2020). Hence national as well as international actions are necessary to prevent further environmental, social and economic impacts (CBD 2008). Many species have been introduced intentionally (Kowarik 2003; Hulme et al. 2008; Lambdon et al. 2008), but also unintentional introduction, release or escape and spread play an important role, especially in semi-natural habitats (Pyšek et al. 2011). In Europe, the importance of pathways differs largely among taxonomic groups. But ecological impacts in plants are much more frequent in intentionally introduced species than in those unintentionally introduced as contaminant to goods and commodities. Similarly, intentional release is the most important pathway for fish, while unintentional introductions are much rarer (Rabitsch et al. 2013; Nehring and Steinhof 2015). Also, for most taxa impact increases with the number of pathways with which a species is associated (Pergl et a. 2017).

The framework for pathway classification originally suggested by Hulme et al. (2008) has been adopted by CBD (2014) and has thus become a global standard for pathway classification. Pergl et al. (2020) tested this framework on European species and found it to be robust, though simple modifications are recommended to improve its usability. In addition to the knowledge base of Hulme et al. (2008), extensive data bases for other taxa were established, such as for forest pathogens (Santini et al. 2013) and marine alien species (Katsanevakis et al. 2013).

With respect to the targets of the CBD, EU Regulation 1143/2014 aims at managing invasive alien species of Union concern, preventing their further spread as well as covering early detection and rapid response at European level. Achieving this objective, various restrictions are set for intentional introductions of these species (Article 7 of EU Regulation), which was requested and appraised by scientists a while ago (Hulme et al. 2009). Especially with regard to unintentional introductions, escape or release of these species into nature and subsequent spread, each member state has to establish an action plan to manage the responsible pathways (Article 13). The goals of the action plan are to (1) identify the pathways which require priority action ('priority pathways') in the member states' territory and marine waters, because of the volume of species or of the potential damage caused by the species entering the Union through those pathways and (2) to implement appropriate measures to prevent unintentional introductions, escape and spread along these pathways.

Within three years of the adoption of the Union list, each Member State shall establish and implement one single action plan or a set of action plans to address the priority pathways it has identified. Recently, the first German action plan has

been adopted by the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety taking into account the legal requirements according to the Federal Act on the Protection of Nature (BMU 2021). Here, we describe the contents of Germany's action plan as well as the method and cooperative process of its development.

Methods

Prioritisation of pathways

To address the first goal of the EU regulation, a previous study analysed 37 pathways of unintentional introduction and spread of invasive alien species into or within Germany and ranked them according to their species volume, i.e. the number of species per pathway (Rabitsch et al. 2018). For the prioritisation, Rabitsch et al. (2018) took into account (a) the species of Union concern as well as (b) the species that are categorised as invasive in Germany by the Federal Agency for Nature Conservation. However, according to Article 13 para 1 of the EU Regulation, the prioritisation of pathways should be based on the species of the Union list only (option (a) above), which included 37 species at the time when the study was prepared by Rabitsch et al. (2018). Given the small number of this selection, especially when compared with the number of alien species in Europe, about 14,000 (Roy et al. 2019), there was a risk of obtaining an inaccurate picture of the pathways' importance. Marine species, for instance, and important pathways in this context were underrepresented on the Union list. To account for this, in addition to the 37 species of Union concern, the analysis has been performed using a wider selection, namely all terrestrial, limnic and marine species expertly listed as invasive in Germany (93 species) (Rabitsch et al. 2018). This latter group also included all 12 species of the first extension of the Union list, which entered into force shortly after the pathway analysis had been completed. It even included several species of the second and third update of the Union list, although their listings only came into force in 2019 and 2022 respectively. In total, 130 invasive species have been analysed for the pathway prioritisation. Resulting from this was a list of 14 priority pathways which was used as a basis for developing an action plan for preventing introductions to, and spread within, Germany. It is debated, though, whether all invasions in all countries can be mapped onto the existing CBD pathway scheme (Faulkner et al. 2020). Still, with slight modifications, this framework seems suitable for Europe (Pergl et al. 2020). Accordingly, Rabitsch et al. (2018) used a slightly adapted version of the CBD scheme considering the pathways for each of the invasion stages introduction, escape or release, and spread. In our project some pathways had to be redefined and combined, to facilitate the assignment of pathways to relevant stakeholder groups (Box 1).

Box 1. Identified priority pathways for the introduction and spread of invasive alien species in Germany. The categories 1 to 4 follow the CBD pathway classification (UNEP 2014).

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1) Escape from confinement
- Botanical gardens
- Ornamental plants
- Pet trade/aquaristics/terraria/ornamental animals
- Zoos (enclosures, public aquaria)

2) Contamination of transported goods
- Contamination of soil, gravel, dead plants (e.g. hay, straw) or similar material (e.g. in earthworks or landscaping)
- With material from garden centres and tree nurseries (e.g.potting compost) in or on plant bio-vectors

3) Stowaways in or on means of transport
- Ballast water
- Fishing and angling accessories
- Growth/accumulation on the hull of ships
- In or on devices/machinery/equipment
- In or on humans or in their luggage (incl. tourism)
- In or on motor vehicles (along roadways)
- In or on trains (along rail-way lines)

4) Unassisted (corridor)
- Unassisted dispersal along canals or waterways between river basins, lakes and seas

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Development of the action plan

For the development of the proposed measures a step-by-step approach was used. Special attention was paid to build on already existing experiences in Germany, the European Union and worldwide concerning the prevention of unintentional introduction and spread of invasive alien species. Further, existing structures, such as federal or Länder (state) ministries, established public relations measures and existing associations were preferably addressed in this first action plan. These structures were used as they can be more easily addressed and monitored than private institutions; furthermore they are more realistic to leverage for pathway management than as yet non-existent structures and activities.

This resulted in a five-step process (Fig. 1): (1) We started the process with a broad literature review (considering studies from all over the world while focussing on the feasibility in Germany) to collect existing and proposed new approaches in pathway management of invasive alien species. (2) Expert consultations were carried out with stakeholders of all prioritised pathways and affected sectors. (3) The resulting list of proposed measures was subjected to a process of selection and aggregation, using a criteria catalogue for the prioritisation (see below). This led (4) to 24 proposed measures and (5) a deliberation process.

(1) Literature review on pathway management

As a first step, national and international measures and code of conducts for the management of the prioritised pathways were reviewed in 2018 and checked for their relevance and applicability for national concerns. We focused on national actions that were already implemented, in order to allow using existing structures and experiences. We also reviewed measures in countries and regions that are especially affected by
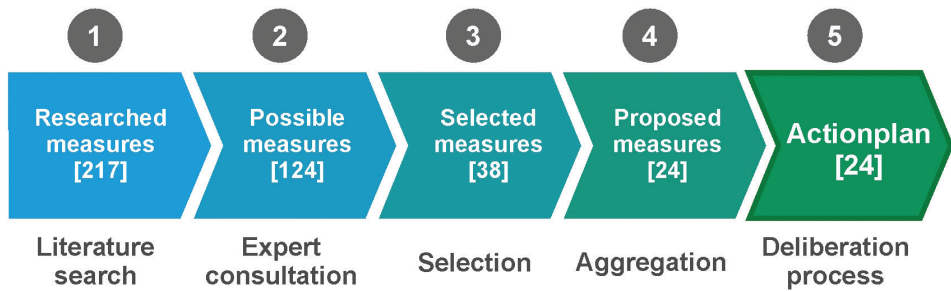
Figure 1. Consecutive steps in the development of the catalogue of measures for the first German action plan on the pathways of invasive alien species. The number of measures resulting from each step is given in square brackets. Thirty-eight out of the 124 possible measures were selected, aggregated and finalised to a list of 24 tangible measures that result in the action plan after a series of deliberation processes.

alien invasions (such as South Africa, Hawai'i and New Zealand). We searched for these plans and other directing measures by scientific databases (Web of Science) and other search engines using keyword combinations such as "action plan", "invasive alien species", "non-native", "biosecurity strategy", "weed and pest", "strategic plan" and other sector- and pathway-specific keyword combinations such as "ornamental plants", "contamination", and "ballast water" as well as backwards search of known documents. Then, we expanded the review on measures that were proposed in the literature but that were not implemented yet. Finally, the resulting list of potential measures was examined for gaps in the coverage of the prioritised pathways. This resulted in 217 measures to be investigated (Fig. 1).

(2) Expert consultations

As a second step, in intense consultations with experts of all affected sectors, the potential measures identified by the literature review were discussed to further develop, prioritise and substantiate measures as well as to discuss ideas for further measures. These consultations thus allowed to incorporate existing experience and knowledge into the action plan.

Nineteen sectors (such as agriculture, conservation, and transport) were identified for being responsible in implementing these actions i.e. were involved in pathway or species management (for a complete list of all sectors involved see Suppl. material 1). For each of these sectors associated experts were involved.

The experts comprised a heterogeneous group that included officials from various ministries, representatives from NGOs, registered associations, think tanks, coordination centres for invasive alien species, working groups, professors, and other specialists such as biologists from universities and various federal and private research institutions, laboratories, councils, state offices, airports, and transportation groups.

The number of experts consulted for each pathway analysis varied between 6 and 30, with an average of 11 experts being consulted per pathway. This was due to the

varying numbers of stakeholders involved in managing invasive alien species that were unintentionally introduced or spread along each pathway.

Additionally, the extent of prior actions taken, as well as the level of awareness and sensitization, varied notably among the different pathways. Some experts were consulted for only one pathway, while others were consulted for up to five pathways in a single session. Consequently, the duration of the individual consultations varied, ranging from 30 minutes to over two hours.

Experts were either interviewed bilaterally, or via individually prepared questionnaires. In total, 62 bilateral talks and 49 individual questionnaires were taken into account. Some discussions with experts were continued in follow-up consultations, in some cases spanning several months. The entire process was done in close cooperation and coordination with the German Federal Agency for Nature Conservation (BfN) and the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU). Drafting the measures was also supported by a project-accompanying working group (PAG) with representatives from authorities, associations and science headed by the BfN, which met twice in Bonn. It proved very useful to bring in all sectors and stakeholders, not only for best exploiting existing knowledge, but also for preparing the ground for efficient and effective implementation.

(3) Selection of measures

An extensive list of 217 possible measures for preventing unintentional introduction and spread of invasive alien species along the priority pathways resulted from the literature review. Consultations with experts reduced this number to 124 measures whose application could be considered and should be discussed more intensively with regard to a manageable implementation in Germany (Fig. 1). This is even more important, due to the given limited German experience in the management of unintentional introduction and the spread of invasive species.

Through dialogue with experts, it turned out that the priority pathways and the respective possibilities for preventive measures differed in many respects, e.g. their degree of implementation. We therefore needed to assess each pathway individually rather than in a bulk approach.

To select a set of manageable measures from the list of 124 measures we performed a step-wise approach according to previously defined criteria (Table 1): Firstly, we determined already existing structures and activities at national or international level in the subject area, on which our potential measures could be based or linked to. Care was taken to explicitly keep those measures that build on experiences and (legal) regulations in Germany and the European Union (e.g. existing self-commitments by professional associations).

Secondly, we estimated the cost-benefit ratio for each suggested measure in accordance with Art. 13 Para. 4 EU Regulation. Measures that could make use of existing workflows, thus not needing extensive additional funds, and at the same time expected to have a broad and long-lasting impact, were given highest priority.

Table 1. Criteria for prioritisation of possible measures.

Builds on existing structures	High priority for measures that build on existing structures, regulations, recommendations, actions or activities in Germany, Europe or internationally
Cost-benefit ratio	High priority of measures with high benefit at low to medium costs
Costs low	Existing resources / personnel structures are sufficient
Costs intermediate	One-time additional funds required
Costs high	Permanent establishment of additional personnel structures and funds required
Benefit low	Local, short-term impact
Benefit intermediate	Regional, medium-term impact
Benefit high	National, long-term impact
One-off or long-term effect	Avoidance of one-off effects; if possible reformation of measure to achieve long-term effect
Avoiding possible conflicts with nature conservation	High priority for measures without potential conflicts with other nature conservation objectives
Synergies with other measures	Actions facilitating each other, within or between different pathways, were given priority

Additional selection criteria were the sustainability of a measure, the avoidance of conflicts with nature conservation, and synergies between different measures. Information, experiences and findings from the bilateral talks on the feasibility of a measure were taken into account during this process (see Table 1). Measures that did not meet or only partially met either of these criteria were either further adjusted or, where this was not possible or sensible, sorted out.

For example, for the pathway "fishing and angling accessories" one suggested measure was to build Crab barriers. During the selection process, however, it turned out that the respective costs would be very high. Further, this measure would not yield an additional nature conservation value, and the responsibilities were not clear. Therefore, this measure was not selected for inclusion in the action plan.

(4) Aggregation

The prioritisation resulted in up to four sensible and possible measures for each pathway (altogether 38 measures). Measures that were not selected were documented in a table for future revisions of the action plan (see Mayer et al. in press). These resulting 38 measures were aggregated into 24 measures (Step 4, Fig. 1). The aim of this aggregation process was to develop actionable sets of measures with clear assignment to specific stakeholders.

(5) Deliberation processes

After a set of measures was selected in step three and compiled into actionable sets of measures in step four, this list became the first draft of the action plan and had to go in step five through a number of deliberation processes according to the Federal Act on the Protection of Nature (BNatSchG), in which all the requirements from the EU Regulation are transposed into German law. As part of the procedure for drawing up the action

plan, the public was involved in accordance with § 40f BNatSchG: The draft of the action plan was available on a website of the BMU in September 2020 for a period of one month for public commenting. This resulted in 73 suggested changes, all of which were evaluated and 25 changes were subsequently incorporated into the draft action plan. A large proportion of the comments received related to the definition of responsibilities and the binding nature of measures. Above all, there were calls to introduce stricter controls and enforcements, and the measures were criticised for being formulated too loosely.

The revised draft of the action plan according to § 40d Abs. 1 BNatSchG was then decided and published by the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU 2021) as a legally binding document after hearing the federal states in agreement with the Federal Ministry of Transport and the Federal Ministry of Agriculture.

Results

Structure of the German action plan

According to Article 13 para 2 of the EU Regulation, each Member State shall establish and implement one single action plan or a set of action plans to address the priority pathways. To make the best use of synergies among several pathways, stakeholders and some cross-pathway measures, we decided to develop one single action plan that addresses each prioritised pathway individually, rather than a set of action plans, i.e. one for each sector. A template was developed giving basic information for each pathway in a header, followed by short descriptions of required measures. In the head section, invasive alien species of Union concern that were, or could be introduced by, or spread along each priority pathway, are listed and involved sectors are mentioned. Subsequently, the overall aim of the measures is described.

The structure and content of the action plan are designed to meet the requirements of the EU Regulation. The description of single measures contains information about: (1) the targeting categories (according to Article 13 para 4 a-c) of the EU Regulation (i.e. raising awareness, minimizing contamination or border checks), (2) the specific aim of the measure, (3) responsible stakeholders and cooperation partners, (4) the target group, (5) a specific description of the measure (according to Article 13 para 2), (6) a rough cost-benefit analysis (according to Article 13 para 4), (7) a time table (according to Article 13 para 2) and (8) a paragraph about the documentation of the measure, since the action plan has to be revised at least every six years (according to Article 13 para 5).

Content of the German action plan

A list and short description of all 24 measures of the German action plan is given in Table 2. The complete action plan is currently only available in German (BMU 2021). Of the 24 specific measures, 16 are cross-sectoral and eight address stake-

Figure 2. Examples of issues addressed in the first German action plan on the pathways of invasive alien species **A** in or on humans or in their luggage (M20) **B** in ballast water of seagoing and inland ships (M13, M14) **C** in or on trains (along railway lines) (M21) **D** growth/accumulation on the hull of ships (M15, M16) **E** contamination of gravel (M10, M11) **F** unassisted dispersal along canals or waterways between river basins, lakes and seas (M22, M23, M24) **G** botanical gardens (M1, M2). M# means Measure number in Table 2. Photo credits: Tina Heger (**D**), Katharina Mayer (**C, E**), Stefan Nehring (**A, B, F, G**).

holders in only one sector. Four measures were laid out across different pathways. 50% of the measures have the aim to raise awareness, which is achieved by (a) public relation activities and by (b) educating and training relevant stakeholders on how species are spread along the prioritised pathways and possible consequences. The other 50% of the measures intend to minimise contamination of goods, commodities, vehicles and equipment by specimens of invasive alien species, including measures to tackle transportation of invasive alien species from third countries, which is done by (c) developing and publishing technical documents or by (d) addressing the need of targeted research projects. Examples of issues addressed in the action plan are illustrated in Fig. 2.

All measures build on existing structures, and the content was discussed and adjusted in accordance with individual stakeholders. The agreement of the stakeholders was seen as an important requirement for a successful implementation of the action plan.

As described above, this was done through a political deliberation process and by interacting with the stakeholders potentially involved in each of the considered measures.

Table 2. List and description of all 24 measures of the first German action plan on the pathways of invasive alien species. M: Measure; CPM: Cross-pathway measure.

Measure #	Pathways	Content	Stakeholders
M1	Botanical Garden	Application and further development of the "Principles for handling invasive and potentially invasive plant species in botanical gardens"	Association of Botanical Gardens in Germany
M2	Botanical Garden	Educating the public: public relation activities for visitors of the botanical gardens	Association of Botanical Gardens in Germany
CPM3	Pet trade/aquaristics/ terraria/ ornamental animals; Ornamental plants; In or on humans or in their luggage	Continuation and further development of web pages	Nature conservation authorities at various administrative levels
M4	Pet trade/aquaristics/ terraria/ ornamental animals	Implementing the "European code of conduct on pets and invasive alien species"	Pet trade and pet store (incl. online trade)
CPM5	Pet trade/aquaristics/ terraria/ ornamental animals; Ornamental plants; With material from garden centres and tree nurseries/ In or on plant bio-vectors	Invasion risk assessment of alien species	Federal Agency for Nature Conservation (BfN)
M6	Zoo (enclosures, public aquaria)	Raising awareness among professionals in animal care training	Bodies responsible for education in the Federal Ministry for Economic Affairs and Energy, ministries for education of the Länder, professional association of zoo keepers (BdZ e.V.)
M7	Zoo (enclosures, public aquaria)	Taking the European Code of Conducts on invasive species in Zoos into account	Animal parks, zoos, enclosures, public aquaria
CPM8	Ornamental plants; With material from garden centres and tree nurseries/In or on plant bio-vectors	Raising awareness among professionals in vocational trainings of gardeners, agriculturists and foresters	Bodies responsible for education in the Federal Ministry of Food and Agriculture; ministries for education of the Länder
M9	Ornamental plants	Application and further development of the recommendations for "handling invasive species" of the German Horticultural Association	German Horticultural Association (ZVG e.V.)
M10	Contamination of soil, gravel and dead plants	Consideration of the topic "minimising the introduction and spread of invasive species via contaminated material" in guidelines and working aids for sustainable building	Federal Ministry of the Interior, Building and Community (BMI) and Federal Office for Building and Regional Planning (BBR)
M11	Contamination of soil, gravel and dead plants	Educating the public and specialists about the proper disposal of green waste, garden waste and soil contaminated with invasive species	Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU), German Environment Agency (UBA), competent Länder authorities, Federal/Länder working group on waste (LAGA)

Measure #	Pathways	Content	Stakeholders
M12	In or on motor vehicles (along roadways)	Considering the handling of invasive species in technical documents for the planning, creation and maintenance of roadside green	Road construction administrations of the federal and Länder governments
M13	Ballast water	Examination of the Ballast Water Management Convention as part of the Experience Building Phase (EBP)	Federal Maritime and Hydrographic Agency (BSH), Federal Ministry of Transport and Digital Infrastructure (BMVI)
M14	Ballast water	Research on the transport and prevention of the introduction, escape or release of alien aquatic species with ballast water in inland navigation	Network of Experts from the Federal Ministry of Transport and Digital Infrastructure (BMVI, BSH, BfG)
M15	Growth / accumulation on the hull of ships	Raising awareness of pleasure craft owners	Diverse professional associations of boating and pleasure crafts
M16	Growth / accumulation on the hull of ships	Research on the transport of alien aquatic species by fouling on ship hulls	Network of Experts from the Federal Ministry of Transport and Digital Infrastructure (BMVI, BSH, BfG)
M17	Fishing and angling accessories	Development of a guideline for dealing with alien species in fishing activities	German Fishing Association (DAFV) and other fishing associations of the Länder
M18	Fishing and angling accessories	Consideration and further development of guidelines in dealing with alien species in aquaculture (edible and stock fish production)	Federal and Länder fisheries authorities, fisheries associations
CPM19	In or on devices / machinery / equipment	Raising awareness among specialists and the public	Respective stakeholders of correspondent actions
M20	In or on humans or in their luggage	Public-relations activities	Federal Agency for Nature Conservation (BfN), Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU)
M21	In or on trains (along railway lines)	Consideration and further development of technical documents for dealing with invasive species regarding the creation and maintenance of greenery along railway lines	German Railways and other railway transport companies
M22	Unassisted dispersal along canals or waterways between river basins, lakes and seas	Raising awareness among specialists	Federal / Länder Working Group on Water, Federal Water Management Associations
M23	Unassisted dispersal along canals or waterways between river basins, lakes or seas	Consideration of the handling of alien animal and plant species in technical documents of the water management, as well as the waterway and shipping administration	Federal / Länder Working Group on Water, Federal Water Management Associations
M24	Unassisted dispersal along canals or waterways between river basins, lakes or seas	Research on migration barriers and technical barriers in shipping canals	Federal Agency for Nature Conservation (BfN)

Discussion

The first German action plan contains measures to raise the awareness of public and specialised staff as well as measures to minimise the contamination of goods, commodities, vehicles and equipment by specimens of invasive alien species, including measures to tackle transportation of invasive alien species from third countries. The first and largest category, raising awareness, covers multiple public relation activities and further education of relevant stakeholders. The second category, minimizing contamination, contains the (further) development of technical documents and calls for research projects. With these proposed actions, the German action plan aims at managing the priority pathways of unintentional introduction and spread of invasive alien species, as required in response to EU Regulation's Article 13.

In accordance with EU Regulation 1143/2014, the management of single species is not the purpose of the German action plan. Neither is its aim to prevent deliberate introductions. This very important group of causes for biological invasions has to be tackled urgently, but action plans based on Article 13 of the EU Regulation are not the appropriate tool for this task. Specific pathway management could be a powerful lever for preventing the unintentional introduction and spread of alien species. The advantage of addressing the pathway instead of single species clearly is that respective measures can affect both known as well as yet unknown invaders spreading along that pathway. It remains to be seen whether the first German action plan is such a powerful lever for efficient prevention of the unintentional introduction, escape or release and spread of future invasive species.

Internationally, actions plans have been published and implemented for several decades. Within the last ten years the number of actions plans worldwide increased tremendously. As part of the literature review we surveyed 56 actions plans for potential and feasible actions in Germany, all published within the last twenty years. These action plans differ in their structure and focus (see Suppl. material 2). Regarding EU Regulation 1143/2014 on invasive alien species, the French action plan was the first, which was uploaded to the EIONET Reporting Obligations Database (ROD). In the meantime, the action plans of all member states will most likely have been published, however, generally in the respective national language. No formal exchange about the development and content of the action plans with other countries of the European Union has taken place. Hence, the conscious development of Europe-wide, synergetic effects was not possible for the first action plan.

However, since regular updates of the action plans are obligatory (the next one for all Member States is due in 2025), a cross-European cooperation should be considered for the future. One reason for the national approach taken during this initial process of developing an action plan was to build on existing national structures, which is most likely also valid for other similar national enterprises. The German action plan, hence, addresses in most cases specific stakeholders, with whom consultations and coordination had taken place beforehand. Regular updates (of the Union list as well as further developments of management techniques), also prevent invasions being

taken as a static event, but allow the dynamic response to changes in invasion and introduction dynamics.

In an exemplary short comparison of the German with the French action plan (Ministère de la Transition Ecologique 2022), different approaches but also commonalities become apparent, so that a desirable EU-wide harmonisation of specific measures seems desirable in the future. The French Action plan consists of 36 measures, structured in one table, focussing on four different topics: (1) Transversal measures, (2) Ornamental and horticultural use, (3) Domestic detention of invasive animals and (4) Aquatic and terrestrial corridors. Each of these themes contain five main operational tools: (A) Raising awareness, (B) Communication and training, (C) Management tools and actions such as legislative and regulative, (D) Control, biosecurity and surveillance such as guides to good practices and codes of conducts, (E) Research, expertise and knowledge. Ten columns describe the single measures, such as: operational tools, a title, spectrum of species and environments, the overall goal, context, target partners and stakeholders, description of the measure, associated actions, similar programs, timeline, priority, cost and the acceptability of the target actors. Hence the measures address raising awareness, and minimising contamination of goods as well as ensuring appropriate checks at the Union borders. The latter is missing in the German action plan. Compared to the German action plan, some measures of the French action plan are more binding. Some French measures include potential options and good practice examples added for better comprehension of the tasks. Target partners are sometimes precisely defined but sometimes also only broadly mentioned.

The EU Regulation describes that binding as well as voluntary measures shall be adopted (Article 13 para 2). The German action plan holds a mainly broad scope of measures and allows stakeholders to decide whether and how to implement their content. Nevertheless, stakeholders should document if and why certain measures have been implemented. Further, the German action plan has not addressed aspects like enforcement, control, border surveillance and biosecurity that are mentioned in several other action plans, for example in Australia and New Zealand (see Suppl. material 2), where border control and enforcement of certain measures are clearly defined in the action plan.

For instance, the first German Action Plan does not, in addition to the official controls by customs pursuant to Article 15 (see § 51a BNatSchG), specify any other appropriate checks at the Union borders as listed in the EU Regulation (Article 13 para 4 c). The extent to which stronger controls at the German borders are necessary should be reassessed in future action plans. In comparison to other national action plans which are more binding as well as precise (listed in Suppl. material 2), the first German action plan strongly counts on voluntary measures. During the drafting process it became obvious that in the given context legally binding initiatives are extremely complex and can only be realised later. These "shortcomings" of the German action plan were also identified during the deliberation process (public participation and the consultation of the Länder). However, the update of the Action Plan, which is currently being developed, will hopefully incorporate these suggestions.

Conclusions and future options

The action plan was adopted in a codified political deliberation process after participation of the public and consultation with the Länder by the responsible Federal Ministry for the Environment in agreement with two other Federal ministries in the sectors of agriculture and transport. Clearly, in democratic societies such political processes are of major importance. Science can offer advice, but the final decisions on which measures will be taken must take into account other arguments as well. These first measures, however, will help preventing the introduction and spread of alien species in Germany, and with future revisions, the action plan can constantly increase its effectiveness. Here, from a scientific point of view, particular attention should be paid to developing and establishing stronger and more binding measures.

This includes ensuring that, as far as required, appropriate checks at Union borders other than the official controls pursuant to Article 15 are additionally implemented (see EU Regulation 1143/2014 Article 13 para 4 c). Since a preventive approach regarding invasive alien species shall consider potential future invasions, it has to be discussed and eventually defined which kind of species, other than the species of Union concern shall be addressed by the action plan in the future. For this, a possible future adoption of a national list of invasive species, as specified in the EU Regulation (Article 12) and in the German Federal Nature Conservation Act (§ 54 para 4), could be helpful. This would further improve the protection of biodiversity in Germany.

In this context, the nature conservation risk assessments for alien species published by the BfN (e.g. Essl et al. 2011; Nehring et al. 2013, 2015; Rabitsch and Nehring 2022) could provide the necessary information for defining invasive species of national importance (Köck 2015).

Another promising approach in reducing future invasions is a closer cooperation with horticulture and pet trade. Labelling and, regarding the former, an increased supply and marketing of native plants have great potential (e.g. Humair et al. 2014). In this context it is important to note that the BNatschG does not aim to only protect biodiversity from invasive species but also promote the integrity of genetic diversity by allowing the planting and sowing from native provenances of plants in the open landscape, only, although exemptions are possible in certain cases (§ 40 para 1 No 4 BNatSchG; Skowronek et al. 2023). In addition, online trade, contributing significantly to the dispersal of ornamental plants worldwide, should be addressed more specifically (Humair et al. 2015).

Moreover, a European-wide cooperation in the revisions of the action plans should be envisioned. Strategies within the country as well as promising approaches on a conservation as well as political level could be exchanged, fostering broader trans-national cooperation. Such cooperation, coordinated by a European centre, was already suggested by Hulme et al. (2009).

Another future goal should be closer cooperation between different stakeholders as well as departments in order to tackle potential invasions risks before they become unmanageable (McNeely et al. 2001). Since in many cases costs amortize over time (Surkov et al. 2008; Richter et al. 2013), it is recommended to focus on opportunities

and cost-effectiveness of the proposed and future measures of the action plan rather than on the first upcoming costs. Closer cooperation as well as straightforward communication among decision-making authorities, funding authorities and other stakeholders, could be one way to go forward. Another option may be the setup of one national funding scheme for the prevention of invasive alien species, independently on the sector of expenditure.

Lastly, the current action plan was only the first one with a clear mandate to analyse existing pathways, i.e. having a more hind-sighted perspective. The updates need to anticipate future developments (e.g. Seebens et al. 2020; Roura-Pascual et al. 2021) and be more fore-sighted.

Acknowledgements

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Supplementary material 1

19 sectors identified and assigned to the 14 prioritised pathways

Authors: Katharina Mayer, Tina Heger, Ingolf Kühn, Stefan Nehring, Mirijam Gaertner

Data type: docx

Link: https://doi.org/10.3897/neobiota.89.106323.suppl1

Supplementary material 2

List of some countries or regions that have already implemented action plans or similar documents

Authors: Katharina Mayer, Tina Heger, Ingolf Kühn, Stefan Nehring, Mirijam Gaertner

Data type: docx

Explanation note: Many international action plans are structured tabularly, sequencing according to the objectives or operational tools. The table describes some examples in more detail.

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DISCUSSION PAPER

Cortaderia selloana, an example of aggressive invaders that affect human health, yet to be included in binding international invasive catalogues

Diego Liendo[1], Juan Antonio Campos[1], Alberto Gandarillas[2,3]

1 *Department of Plant Biology and Ecology, University of the Basque Country (UPV/EHU), 48080 Bilbao, Spain* **2** *Institute for Research Marqués de Valdecilla, Ave. Herrera Oria SN, 39011 Santander, Spain* **3** *INSERM, Languedoc-Roussillon, 34394 Montpellier, France*

Corresponding authors: Diego Liendo (diego.liendo@ehu.eus); Alberto Gandarillas (agandarillas@idival.org)

Abstract

Invasive plant species can suppress local biodiversity, affect soil properties and modify the landscape. However, an additional concern of plant invasions that has been more disregarded is their impact on environmental human health. Here, we discuss the case of *Cortaderia selloana* (Schult. & Schult.f.) Asch. & Graebn, as an example of a worldwide invasive species with a strong environmental impact. We summarise the main facts regarding the *C. selloana* invasion, the recent clinical evidence of its impact on human health and the great potential expansion of the species in the context of climate change. *C. selloana* constitutes a clear example to boost demands from policy makers for urgent and efficient measures to control or eradicate invasive species, also in ruderal areas. This aggressive invader is still out of relevant binding international invasive species catalogues, including the European List of Invasive Alien Species of Union concern (Union list), and is still subjected to extensive trading in some European countries. Therefore, including *C. selloana* in the Union list becomes mandatory to impose full restrictions on keeping, importing, selling, breeding and cultivating the species.

Keywords

Introduction

Biological invasions pose a substantial threat to ecosystems worldwide and constitute a critical driver of global change (Vilà et al. 2011). Invasive species are a global concern due to their negative ecological impacts, which include the loss of biodiversity at local and regional scales (Manchester and Bullock 2000; McGeoch et al. 2010; Vilà et al. 2011) or changes in community structure (Domènech et al. 2006; Hejda et al. 2009). In addition, invasions are associated with important economic and human-welfare impacts (Pimentel et al. 2000; Mazza et al. 2014; Hoffmann and Broadhurst 2016). For instance, the economic costs of invasive alien species in Europe have been estimated to total €116.61 billion between 1960 and 2020 (Haubrock et al. 2021). However, beyond the damage to ecosystems and the economy, we need to address the impacts of plant invasions on human health. Unlike invasive animals, the impacts of invasive plants on human health in Europe have been scarcely studied so far and only for a very small number of species, such as *Ambrosia artemisiifolia* or *Heracleum mantegazzianum* (Schindler et al. 2015; Bayliss et al. 2017). In this context, the grass *Cortaderia selloana*, commonly known as pampas grass, rapidly emerges as a species with a great ecological impact (Domènech et al. 2006) and with a clear potential impact on human health as a consequence of: (i) its striking current worldwide spread and (ii) the recently unveiled allergenic properties of the pollen (Rodríguez et al. 2021).

C. selloana and the morphologically close *C. jubata* have been traditionally regulated as distinct taxa. However, in a new synoptic taxonomy of the genus (Testoni and Linder 2017) the authors describe *C. selloana* and *C. jubata* as two subspecies within *C. selloana*. Although this could be of some relevance in terms of the potential inclusion of *C. selloana* in binding international invasive catalogues, we will here consider these taxa as distinct species following the previous nomenclature by Ascherson and Graebner (1900): *Cortaderia selloana* (Schult. & Schult.f.) Asch. & Graebn. [Syn. Mitteleur. Fl. [Ascherson & Graebner]. 2(1): 325].

Worldwide spread of *C. selloana*

C. selloana is native to South America and has naturalised in many regions across the globe, mainly Australia, New Zealand, South Africa, North America and Western Europe. In the latter, *C. selloana* has strongly colonised several countries such as Italy and the Atlantic coast including the UK, the Netherlands, Belgium, France, Spain and Portugal (Fig. 1) (Lambrinos 2001; Tarabon et al. 2018). In Europe, *C. selloana* is widely traded and used as ornamental species. This ornamental use includes fencing and protecting the garden or property due to its sharp leaves and decorating purposes with dried plumes. The expansion of *C. selloana* is strongly associated with human activity, as it colonises preferentially anthropogenic ruderal habitats of low environmental value, such as quarries, road margins, slopes, wastelands or industrial areas (Domènech and Vilà 2007; Pardo-Primoy and Fagúndez 2019). However, the species is also invading natural and semi-natural habitats of high environmental value, such as grasslands, wetlands, marshes and coastal dunes (Campos et al. 2004; Saura-Mas and Lloret 2005; Herrera et al. 2017) (Fig. 2).
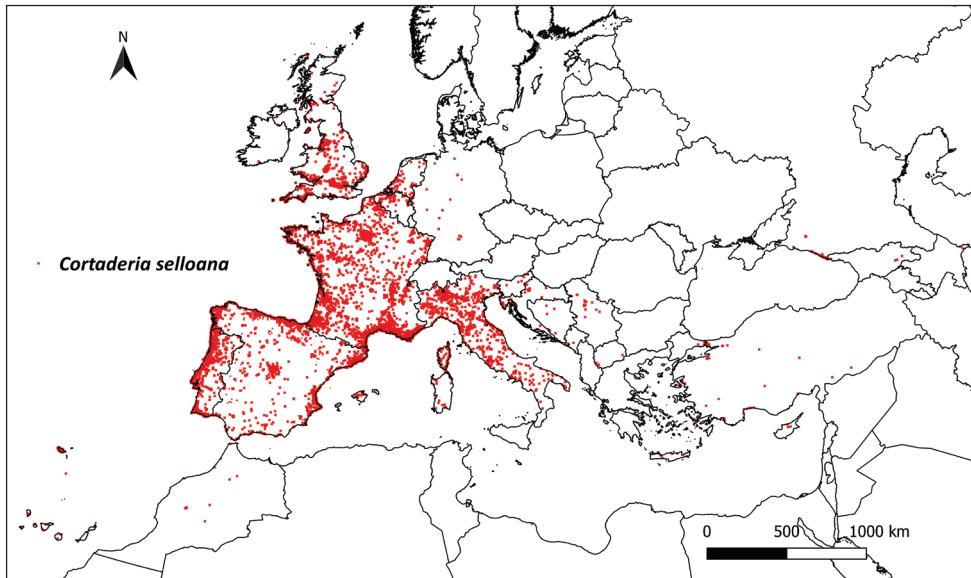
Figure 1. Current distribution of *Cortaderia selloana* (red squares) in Europe and nearby Mediterranean countries. Data retrieved from the Nature Database of Spain (available at: https://www.miteco.gob.es/es/biodiversidad/servicios/banco-datos-naturaleza/) and GBIF-Global Biodiversity Information Facility [GBIF.org (21 September 2022) GBIF Occurrence Download https://doi.org/10.15468/dl.fze4z8].

Impact of *C. selloana* on environmental health

Until recently, skin cuts due to the sharp leaves of *C. selloana* were the only impact associated to this species reported on human health (Mazza et al. 2014). However, grasses are a main source of human allergy (García-Mozo 2017). The potential allergenicity of the pollen of *C. selloana* had been suggested (Street et al. 1979; Başnou 2009; Pyšek and Richardson 2010; Kumar Rai and Singh 2020), but only recently has this allergenic potential been empirically confirmed in a human pilot study conducted in northern Spain (Cantabria; Rodríguez et al. 2021). This study concluded that the grass allergy season in Western Europe might be extended by several months. This first clinical study of the impact of *C. selloana* on human health emphasises that authorities of the international community should run larger clinical allergy tests, especially where it is highly invasive. While the allergenic capacity of *C. selloana* has only recently been demonstrated, a small bunch of other invasive plant species were already known to affect humans. These include giant hogweed (*Heracleum mantegazzianum*) and common ragweed (*Ambrosia artemisiifolia*) (Pyšek et al. 2007; Hemmer et al. 2011; Mazza et al. 2014; Montagnani et al. 2017). However, the potential impacts on human health of many other invasive plant species widely distributed in Europe are far less known. The estimations of the socioeconomic impact of the *C. selloana* invasion and other species should also be re-evaluated considering their impact on human health.

Figure 2. *Cortaderia selloana* can invade natural and anthropogenic habitats **A** Saltmarsh community in the Butrón estuary near Plentzia (Basque Country, Spain) **B** Saja-Besaya Natural Park in the municipality of Cieza (Cantabria, Spain) **C** urban area in Castro Urdiales (Cantabria, Spain) **D** abandoned quarry in Cuchía (Cantabria, Spain).

C. selloana and climate change

Under a scenario of increasing temperatures in the context of climate change, it is expected that plant species will experience spatial shifts in their distribution ranges (Parmesan and Yohe 2003; Rasmussen et al. 2017), reaching regions that currently are climatically unsuitable (Thuiller et al. 2005; Storkey et al. 2014; Rasmussen et al. 2017). This has been tested for some highly allergenic invasive alien plants present in Europe, such as common ragweed, which is predicted to expand its distribution range
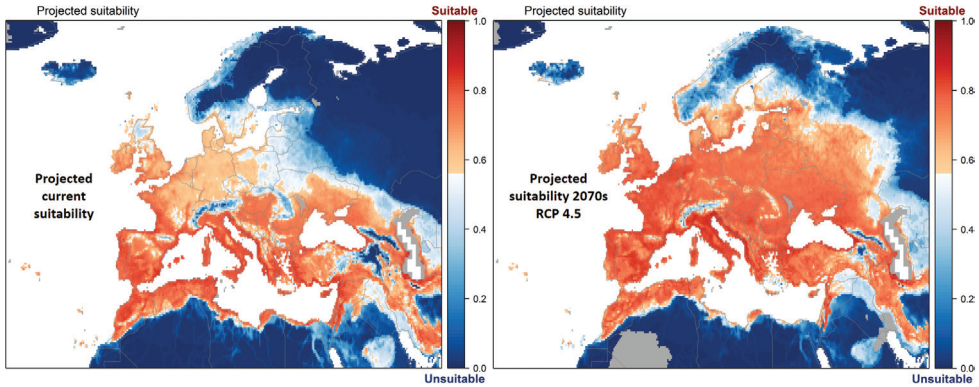
Figure 3. Maps of projected current and future suitability for *Cortaderia selloana* establishment in Europe and other countries of the Mediterranean region. Left: Projected current suitability. Right: Projected future suitability in the 2070s under Representative Concentration Pathways (RCP) 4.5, equivalent to a medium emissions scenario. Source: Brundu et al. (2021).

northwards and eastwards in the forthcoming decades as global temperatures continue to rise (Storkey et al. 2014; Lake et al. 2017; Rasmussen et al. 2017). A similar trend is to be expected with *C. selloana*. Tarabon et al. (2018) reported that while this species already occupies large areas along the western and Mediterranean French coasts, their models predicted an expansion northward and inland to the east under future climates. They define *C. selloana* as a 'climate warming winner.' This suggests that the species may colonise in the future other European regions that currently are climatically unsuitable as temperatures continue to rise, with a potential increase in human health impacts caused by its allergenic pollen.

In this sense, the European Union has recently made a good projection of current and future suitability for *C. selloana* establishment in the EU under current climate and under two scenarios of climate change with similar results (Fig. 3) (Brundu et al. 2021). When comparing the current presence of the species (Fig. 1) with the projected current suitability (Fig. 3), we can see that the southern Atlantic coast has a similar suitability to that of the Mediterranean Basin. Yet, while the presence of *C. selloana* is currently overwhelming on the Atlantic regions it is less abundant on the central-eastern Mediterranean regions (Fig. 1). This is likely due to an initial colonisation of the plant in Europe through Atlantic countries. This means that current conditions might be even better for *C. selloana* in the Mediterranean coasts, especially in humid soils, which predicts a strong invasion in these countries. Regarding the projected maps of suitability in the 2070s based on estimated future climate conditions under the Representative Concentration Pathways (RCP) 2.6 and 4.5, which represent low and medium emissions scenarios, respectively (Brundu et al. 2021), the whole of Europe and the Mediterranean region are expected to be highly suitable for the establishment of *C. selloana* under both scenarios (Fig. 3, RCP 4.5 scenario).

Need for an active EU strategy

In this context, some goverments have begun to take measures against the invasion by *C. selloana*. In some countries, such as the UK or Spain, where the grass is extensively established, it has been officially classified as an invasive species, with prohibition to import, plant, maintain or commercialise (Bayón and Vilà 2019). The species is also in the European and Mediterranean Plant Protection Organization (EPPO) list of invasive alien plants (https://www.eppo.int/ACTIVITIES/invasive_alien_plants/iap_lists). However, the European Union (EU) has not yet included *C. selloana* in the List of Invasive Alien Species of Union concern (the Union list; Regulation (EU) 1143/2014 and subsequent updates), despite having approved in recent years a €3.5 million LIFE project to tackle the invasion in the Western Atlantic coast from 2018 (LIFE Stop Cortaderia, http://stopcortaderia.org/), which has been recently granted another aid of €6.5 million starting in 2023 (LIFE22-GOV-ES-Coop Cortaderia). Other EU projects tackled restoration of natural areas, where *C. selloana* was one of the main invaders (Basic Search on LIFE Public Databases). Consequently, we encourage the European authorities to implement an EU level plan to tackle the invasion by *C. selloana*. Although some local plans are being implemented, a common EU plan would be instrumental. To serve this purpose, a first step should be its inclusion in the Union list, which involves restrictions on keeping, importing, selling, breeding and growing of those species included in the list. This is especially important given that numerous suppliers are listed throughout EU Member states and that both seeds and dried plumes can be easily purchased via online suppliers (Brundu et al. 2021).

The *C. selloana* invasion is not yet as prominent in the Central or Northern EU countries and this might pose some conflict of interest within the Union. However, in the latest risk study report, the European Commission has identified this species with high risk and pointed out that a ban on sale could act to prevent further spread of the species (Brundu et al. 2021). According to the same report, *C. selloana* might be worth more than €200 million every year in Europe as a result of 20–25 million plants being cultivated at European nurseries. Our request to include *C. selloana* in international binding lists is also in line with the conclusions of the IUCN World Conservation Congress held in 2021 in Marseille, France, which included a call on governments in the EU to propose the inclusion of this species in the Union list (https://www.iucncongress2020.org/motion/005). Finally, it can only be paradoxical that *Cortaderia jubata*, a morphologically close species of the same genus not widely present in the EU, was included in the Union list in the second update entered into force on 15 August 2019. If the ecological facts were not sufficiently convincing, the recent findings proving the allergenic capacity of the plant reinforce the urge to implement an EU common plan against the *C. selloana* invasion. Our further conclusive message is that invasive plant species must be fought not only because of their ecological impact, but also because of their impact on human health. This adds another level of concern that will require the involvement of health governmental authorities, agencies, and policy makers.

Acknowledgements

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RESEARCH ARTICLE

Biochar application can mitigate the negative impacts of drought in invaded experimental grasslands as shown by a functional traits approach

Hamada E. Ali[1,2], Solveig Franziska Bucher[3,4],
Markus Bernhardt-Römermann[3,4], Christine Römermann[3,4]

1 *Department of Biology, College of Science, Sultan Qaboos University, Muscat, Oman* **2** *Botany and Microbiology Department, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt* **3** *Institute of Ecology and Evolution with Herbarium Haussknecht and Botanical Garden, Friedrich Schiller University Jena, D-07743 Jena, Germany* **4** *German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, D- 04103 Leipzig, Germany*

Corresponding author: Hamada E. Ali (helsayedali@gmail.com, h.ibraheem@squ.edu.om)

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Abstract

Climate, land-use, and invasive plants are among the important drivers of ecosystem functions through the changes in functional composition. In this study, we studied the effects of climate (drought), land-use (Biochar application), and the presence of invasive species on the productivity and performance of invaded experimental grasslands. We ran a greenhouse experiment under controlled conditions, in which we grew a combination of the three native species *Silene gallica*, *Brassica nigra* and *Phalaris minor* and the invasive species *Avena fatua*, being subjected to four different treatments: Biochar+drought, Biochar, drought, and control. We measured the productivity of native and invasive species as total biomass and root to shoot ratio (RSR) and the performance by measuring several plant functional traits (plant height, specific leaf area (SLA), leaf dry matter content (LDMC), leaf nitrogen content (N_{mass}), leaf carbon content (C_{mass}) and total chlorophyll (Chl_{total}) of all individuals occurring in each plot. The study showed that invasive species were more productive (higher total biomass and lower RSR) and performed better (taller plants, higher SLA, N_{mass}, C_{mass} and Chl_{total} and lower LDMC) than the native species under drought conditions as well as with Biochar application. Accordingly, in contrast to our expectations, the lower productivity and performance of native compared to invasive species under drought were not mitigated by Biochar application. These results provided a deeper understanding of the interplay between climate, land-use, and biological invasion, which is crucial for predicting the consequences of changes in functional composition on ecosystem functions and consequently restoration of grasslands.

Introduction

With ongoing climate change, drought events have become more frequent and severe (Dai 2012) which is affecting species composition, diversity and ecosystem functions in many ecosystems (Jung et al. 2020). Water shortage decreases plant productivity and influences species abundance, plant distributions, community composition (Knapp et al. 2002; Wellstein et al. 2017; Wei et al. 2022) as well as plant phenology (König et al. 2018) and therewith affects biotic interactions (Montoya and Raffaelli 2010). However, factors like human activities, primary land-use changes, being important drivers of global biodiversity in grassland systems, are changing in parallel (Sala et al. 2000). Human activities increase the potential risk of invasion by invasive species, which threatens global biodiversity and is often maximized by changes in climate and land-use. In grasslands, invasive plants can affect the native communities directly by competing for resources (e.g., light, nutrients, water) (Gooden and French 2015; Fristoe et al. 2021; Kühn et al. 2021), by changing the physical structure of the grasslands as diverse grasslands are frequently converted into dense monoculture formed by one invasive species (Guido et al. 2016), and indirectly by altering soil properties (e.g., nutrient availability and soil moisture) (Mahood et al. 2022). This is because invasive plants effectively use empty niches and, once established, outcompete native plants as they tend to have higher growth rates than natives within the same sites (Allison and Vitousek 2004; Ali and Bucher 2021; Kühn et al. 2021). Therefore, understanding the interplay between land-use change, climate change and biological invasion is critical for predicting the consequences of human-induced changes on ecosystem functions (Pejchar and Mooney 2009; Bernhardt-Römermann et al. 2011).

One of the nature-based solution goals of international nature conservation and climate change mitigation is ecosystem restoration (Griscom et al. 2017), which is essential to help ecosystems adapt to adverse impacts of climate change like extreme weather events (Chausson et al. 2020) and benefit biodiversity (Morecroft et al. 2019; Seddon et al. 2021). Among the available tools used in grassland management to restore degraded ecosystems is the application of Biochar, which improves soil conditions after degradation and consequently improve the ecosystem functions (Joseph and Lehmann 2015; Mandal et al. 2016). Biochar is a carbon-rich material produced by biomass pyrolysis or gasification processes in an oxygen limited environment (Lehmann et al. 2015). It enhances soil fertility directly by providing essential soil nutrients and soil carbon (Coomes and Miltner 2017; Igalavithana et al. 2017) or indirectly by neutralizing soil acidity (Zhang et al. 2017) and increasing water holding capacity as well as soil aeration.

The benefits of ecosystem functions and related processes of change may be associated to plant functional traits, such as maximum plant height (H_{max}), specific leaf area (SLA), leaf dry matter content (LDMC), leaf nitrogen content (N_{mass}), leaf carbon content (C_{mass}), total chlorophyll content (Chl_{total}) and root to shoot ratio, which might give valuable insights into ecosystem properties. H_{max} is a good assessment of

competitive strength, as plants compete for light (Pérez-Harguindeguy et al. 2013). SLA is mainly related to growth rates (Garnier et al. 1997; Knops and Reinhart 2000; Hulshof et al. 2013; Pérez-Harguindeguy et al. 2013) whereas LDMC is a measure of investment of the plant in defense and structural components (Pérez-Harguindeguy et al. 2013). Leaf nitrogen reflects the photosynthesis rates as most N in the leaves is located in rubisco, the main enzyme of carbon fixation (Yang et al. 2020). Leaf carbon content is connected to nutrient acquisition (Xing et al. 2021). Chl_{total} reflects plant health, photosynthetic capacity, and nutrient acquisition (Li et al. 2018b). Finally, we studied the root to shoot ratio (RSR), which can be used as a proxy of the plants' ability to tolerate drought (Cambui et al. 2011).

Here, we study the interacting effects of drought, Biochar application as well as the presence of invasive species, on ecosystem processes on Egyptian grasslands. These grasslands are recently threatened by more frequent and severe droughts likely due to ongoing climate change (Asklany et al. 2011; Mossad and Alazba 2015). Overexploitation and the increasing dominance of invasive species such as *Avena fatua* L., cause severe impacts on the local plant communities (Zahran and Willis 2008). *A. fatua* is considered a destructive invasive weed not only to croplands but also of grasslands (Beckie et al. 2012), as it has favorable traits compared to the native species with respect to higher seed production, seed persistence in soil seed bank due to its dormancy, rapid growth, substantial root system, and the ability to germinate under a wide range of environmental conditions (El-Shatnawi and Ghosheh 1999; Beckie et al. 2012; Bajwa et al. 2017). Such traits foster the ability to outcompete several native grasses and consequently affect the entire grassland ecosystem. To assess the impact of drought and Biochar application as a useful tool to mitigate the drought effects on plant functional traits and above and belowground biomass production of invaded grassland communities, we set up artificial grassland communities in a greenhouse consisting of native species mixed with *A. fatua*. We tested the effect of drought and Biochar application in relation to a control treatment on the performance of native and invasive species on germination and growth traits. More specifically, we asked whether the combined effect of drought and Biochar application affect the traits indicating early individual performance as well as productivity of native and invasive species in artificial grassland communities.

The results of this study will provide the basis for deciding whether the restoration of grassland communities affected by drought and *A. fatua* is viable through Biochar application.

Materials and methods

Experimental setup

To test the effects of drought and Biochar application on native and invasive species, we established artificial grasslands in a greenhouse consisting of four annual herbaceous species, three of them natives, namely *Silene gallica* L. (Caryophyllaceae), *Brassica nigra* L. (Brassicaceae) and *Phalaris minor* Retz. (Poaceae) which are frequently coexisting

in the species-poor Egyptian grasslands (Zahran and Willis 2008). We additionally investigated the effect of the invasive *A. fatua* L. (Poaceae). All the species used in the experiment have comparable germination and establishment rates based on our previous knowledge.

On March 21st, 2021, a greenhouse experiment was set up at the Suez Canal University, Ismailia, Egypt (30.6205°N, 32.2697°E) with a temperature maintained between 20 °C and 25 °C. We used a full factorial design (Biochar+drought, Biochar, drought, and control) of a mixture of native and invasive species. We sowed 25 seeds per species (in total 100 seeds) in 0.5m x 0.5m experimental plots (Suppl. material 1: fig. S1) which were filled with soil from the study area within the Suez Canal University Campus. Seeds used in the experiment were collected from the study area in autumn 2020 and viability tests were performed by germinating them on wet filter paper before sowing. There was a total of five replicates for each of the four combinations and, in addition, five plots were left without seeds or treatment to see if any other seeds would germinate from the seedbank, resulting in a total of 25 plots (5 plots × 4 treatments "Biochar+drought, Biochar, drought, and control" + 5 plots without seeds nor treatments) in a random setting within the greenhouse (Fig. 1). For the Biochar application, 10 plots received 1.25 kg of Biochar mixed with the topsoil before sowing; the other 10 plots did not receive any Biochar application (Suppl. material 1: fig. S1). We provided optimal conditions during germination time by sufficiently watering the experimental plots, ensuring 60% of water saturation (= 540 ml per day in the first week, afterwards watering every second day) as recommended by Dietrich et al. (2022).

In April 2021, five similarly sized individuals per species and plot were chosen for the experiment (n = 20 individuals per plot). The remaining seedlings as well as any other species grown within the study plots were removed at the beginning of the experiment.

To simulate the effect of drought, the experimental plots were divided into two watering treatments: the control plots (n = 10) were watered with 540 ml twice a week as before whereas the drought plots (n = 10) were watered twice a week with just 180 ml, which represent 20% of soil saturation after the initial establishing phase following Ali and Bucher (2022).

Functional trait measurements

Before being harvested on November 1st 2021, above and belowground traits (H_{max}, SLA, LDMC, N_{mass}, C_{mass}, Chl_{total} and RSR) were measured following standardized protocols (Pérez-Harguindeguy et al. 2013) on each individual within each plot to account for intraspecific trait variability (Albert et al. 2012; Ali et al. 2017) (Table 1). H_{max} (cm) was measured as the shortest distance from ground level to the highest photosynthetic tissue using a ruler (to the nearest cm). To measure SLA and LDMC, three healthy fully developed and sun-exposed leaves were collected for each individual in each plot and measured together as one pooled sample. SLA, which is defined as the ratio of fresh leaf area (LA) to dry mass expressed as ($mm^2\ mg^{-1}$), was measured by measuring the two leaf dimensions using a ruler (mm), then these two
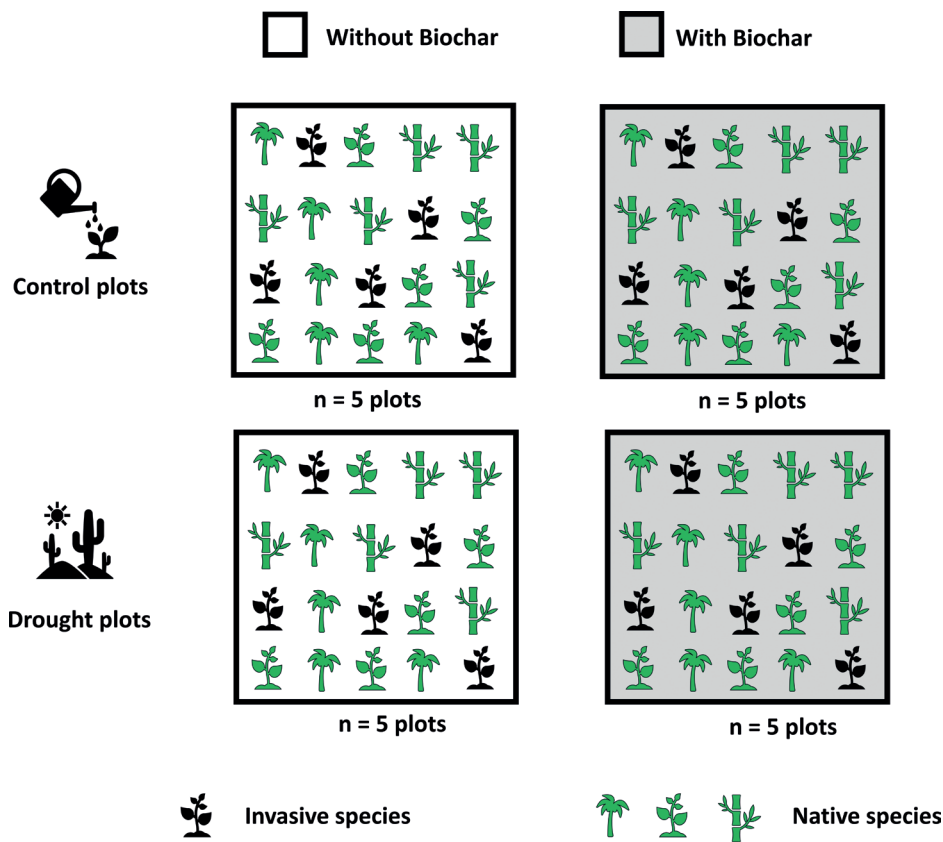
Figure 1. Experimental design to investigate the effects of drought (drought and control) and Biochar application (with and without Biochar). A total of 20 plots were cultivated with five individuals from each of the invasive species *Avena fatua* (black) and the three native species *Silene gallica*, *Brassica nigra* and *Phalaris minor* (green) (in total *n* = 20 individuals / plot). The treatments were Biochar+drought, Biochar, drought, and control (*n* = 5 plots per treatment).

Table 1. List of the measured plant functional traits, abbreviations, measuring unit and their ecological function.

Trait	Abbreviation	Unit	Function	Reference
Maximum plant height	H_{max}	Cm	Light, water and nutrient acquisition, competitive strength	Moles et al. (2009) and Pérez-Harguindeguy et al. (2013)
Specific leaf area	SLA	mm² mg⁻¹	Nutrient acquisition, growth rates	Garnier et al. (2001) and Pérez-Harguindeguy et al. (2013)
Leaf dry matter content	LDMC	mg g⁻¹	Resource use strategy	Garnier et al. (2001) and Pérez-Harguindeguy et al. (2013)
Leaf nitrogen percentage	N_{mass}	%	Photosynthetic capacity and nutrient acquisition	Yang et al. (2020) and Pérez-Harguindeguy et al. (2013)
Leaf carbon percentage	C_{mass}	%	Nutrient acquisition, resistance	Xing et al. (2021) and Pérez-Harguindeguy et al. (2013)
Total chlorophyll content	Chl_{total}	mg g⁻¹	Plant health, photosynthetic capacity, and nutrient acquisition	Li et al. (2018a) and Pérez-Harguindeguy et al. (2013)
Root to shoot ratio (based on biomass)	RSR		Adaptability to dry conditions	Cambui et al. (2011) and Pérez-Harguindeguy et al. (2013)

dimensions were multiplied to get a rough estimation of the total LA (mm^2). The leaves were weighed to record the fresh mass and subsequently oven-dried at 70 °C for 48 h and weighed again to assess the leaf dry mass (mg). Finally, the LA was divided by the leaf dry weight to calculate SLA. In addition to that, LDMC was measured as the dry mass (mg) divided by its water-saturated fresh mass (g), expressed in mg g^{-1}. Moreover, we measured the leaf nitrogen and carbon percentages (N_{mass}, and C_{mass}) on the same oven-dried leaves that were used for measuring the SLA and LDMC as percentage of dry mass in 0.020 g of the milled and dried leaf tissue by using a Perkin Elmer 2400 CHNS Organic Elemental Analyzer. To measure the chlorophyll content of each individual in each plot, 0.1 g of fresh leaves were used to extract chlorophyll using 95% ethanol. The chlorophyll content (Chl_{total}) in mg g^{-1} of the filtered solution was measured using the spectrophotometric method (UH4150AD UV-Vis-NIR Spectrophotometer, Hitachi, Japan) (Mackinney 1941; Li et al. 2018b). For biomass harvest and root to shoot ratio (RSR), the plants were cut at the soil surface, dried at 70 °C for 48 h and weighed as aboveground biomass (g), then the RSR for each individual was measured as the ratio of the root dry weight to the shoot dry weight as described by Mašková and Herben (2018).

Statistical analysis

As a first step, we used a Principal Component Analysis (PCA) to reveal relationships between the plant functional traits per species per plot (H_{max}, SLA, LDMC, N_{mass}, C_{mass}, Chl_{total} and RSR) as well as community-level total biomass production of the four different treatments (Biochar+drought, Biochar, drought, and control) in an ordination plot.

Secondly, we used linear mixed effects models (LMM) to analyze the combined effect of drought and Biochar application on (1) the performance of native and invasive species (H_{max}, SLA, LDMC, N_{mass}, C_{mass}, and Chl_{total}) and (2) the productivity of the invaded plant communities (total biomass and RSR). In both models, productivity or traits at the level of individuals were the dependent variable, the drought (vs. control), Biochar application and the interaction between them were used as explanatory fixed factors and the plot ID was used as random intercept. Restricted maximum likelihood (REML) was used as parameter estimate. Finally, we compared the marginal and conditional R^2 for each model to assess the impact of the random effect as the marginal R^2 is related to variance explained by fixed factors and conditional R^2 is related to variance explained by both fixed and random factors (Nakagawa and Schielzeth 2013).

Finally, to support the interpretation of the data we performed pairwise comparisons using Tukey's post-hoc test to determine if there were differences between native and invasive species under the four different treatments (Biochar+drought, Biochar, drought, and control) for all the measurements.

All statistical analyses were performed using R, version 4.3.0 (R Development Core Team 2023), package "*nlme*" used for the LMMs (Pinheiro et al. 2022) and package "*rstatix*" used to perform the Tukey's pairwise comparison (Kassambara 2023).

Results

Plant functional trait and biomass responses to Biochar and drought

The PCA on species traits and total species biomass showed distinct partitioning of the four treatments (Biochar+drought, Biochar, drought, and control) (Fig. 2). While the first axis seemed to be based on Biochar addition, the second axis represented water availability. Plants grown under the Biochar+drought treatment had a higher SLA, C_{mass} and N_{mass}. Plants that grew in the Biochar only treatment had higher H_{max}, Chl_{total}, shoot and root biomass. Moreover, plants that grew in the control plots showed the highest LDMC. Finally, plants that grew in the drought plots had higher values for RSR (Fig. 2). Interestingly, *A. fatua* showed a rather striking pattern in the drought treatment, which seemed not to have influenced its performance at all (Fig. 2 and Suppl. material 1: fig. S2). Also, in the Biochar+drought treatment it was located more to the left, indicating higher SLA, C_{mass}, and N_{mass}, thus overall higher performance than the native species.

Effects of drought and Biochar on the performance of invasive vs native species

We could confirm the results of the PCA by looking into each trait specifically (Fig. 3a–f, Table 2, and Suppl. material 1: table S1). However, there were no significant differences between native and invasive species for the control treatment except for
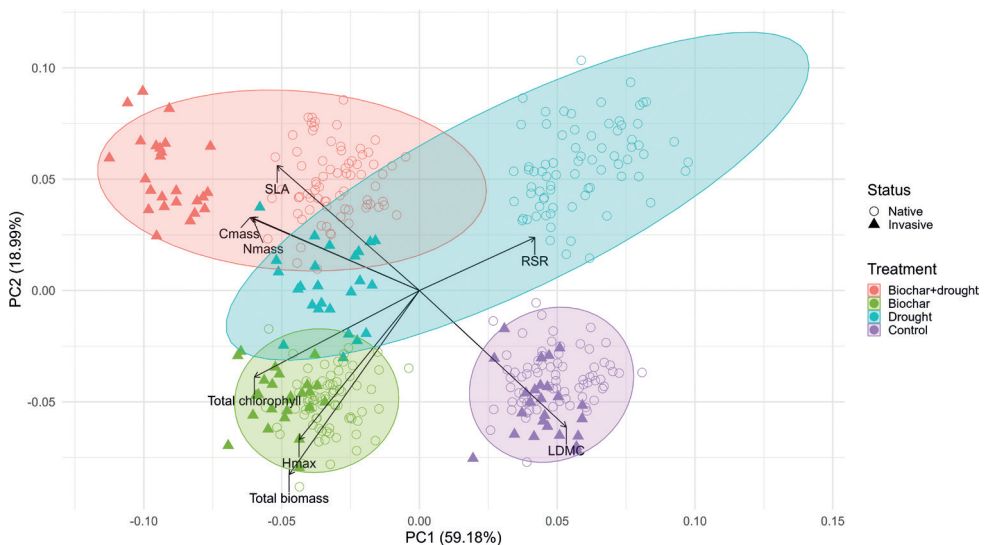

Figure 2. PCA results of the plant functional responses (Maximum height (H_{max}), Specific leaf area (SLA), Leaf dry matter content (LDMC), Leaf nitrogen content (N_{mass}), leaf carbon content (C_{mass}), Total chlorophyll (Chl_{total})) and species production (total biomass and root to shoot ratio (RSR)) of native and invasive species as a response to the four different treatments (Biochar+drought, Biochar, drought, and control).

Table 2. Estimates, standard error (SE), degree of freedom (DF), t-statistics, *P*-values, marginal, and conditional R² for linear mixed effect models testing the effect of drought, Biochar application and the combined effect of drought and Biochar application on the shoot, root biomass and plant functional traits (H_{max}, SLA, LDMC, N_{mass}, C_{mass}, Chl_{total} and RSR). Statistically significant variables are indicated in bold.

Response variable	Explanatory variables	Estimates	SE	DF	*t*-value	*P*	Marginal *R²*	Conditional *R²*
I. Species Performance								
H_{max}	Intercept	57.36	1.08	379	53.36	**<0.001**	0.67	0.67
	With Biochar	28.04	1.47	16	19.11	**<0.001**		
	Drought	-0.08	1.47	16	-0.05	0.957		
	Invasive	17.39	1.13	379	15.42	**<0.001**		
	With Biochar × drought	-25.44	2.07	16	-12.26	**<0.001**		
SLA	Intercept	1.47	0.07	379	20.48	**<0.001**	0.72	0.72
	With Biochar	1.41	0.1	16	14.52	**<0.001**		
	Drought	1.42	0.1	16	14.59	**<0.001**		
	Invasive	1.2	0.08	379	15.1	**<0.001**		
	With Biochar × drought	-0.01	0.14	16	-0.09	0.933		
LDMC	Intercept	463.78	7.31	379	63.45	**<.001**	0.75	0.76
	With Biochar	-119.1	10.16	16	-11.72	**<.001**		
	Drought	-99.07	10.16	16	-9.75	**<.001**		
	Invasive	-58.65	5.37	379	-10.92	**<.001**		
	With Biochar × drought	-5.8	14.37	16	-0.4	0.69		
N_{mass}	Intercept	2.25	0.09	379	24.23	**<.001**	0.77	0.77
	With Biochar	3.07	0.13	16	24.38	**<.001**		
	Drought	1.3	0.13	16	10.36	**<.001**		
	Invasive	0.94	0.1	379	9.13	**<.001**		
	With Biochar × drought	-0.18	0.18	16	-1.02	0.369		
C_{mass}	Intercept	13.69	0.55	379	24.84	**<.001**	0.79	0.79
	With Biochar	17.93	0.75	16	23.78	**<.001**		
	Drought	8.6	0.75	16	11.41	**<.001**		
	Invasive	4.67	0.56	379	8.4	**<.001**		
	With Biochar × drought	-2.37	1.07	16	-2.22	**0.041**		
Chl_{total}	Intercept	7.41	0.16	379	46.62	**<.001**	0.70	0.70
	With Biochar	4.52	0.22	16	20.92	**<.001**		
	Drought	-0.7	0.22	16	-3.25	**<.001**		
	Invasive	2.17	0.18	379	12.3	**<.001**		
	With Biochar × drought	-0.89	0.31	16	-2.91	**0.024**		
II. Productivity								
Total biomass	Intercept	22.66	0.29	379	77.14	**<.001**	0.82	0.82
	With Biochar	7.40	0.34	16	18.52	**<.001**		
	Drought	-8.13	0.34	16	-20.34	**<.001**		
	Invasive	5.28	0.32	379	16.47	**<.001**		
	With Biochar × drought	0.88	0.56	16	1.55	0.1391		
RSR	Intercept	0.57	0.01	379	53.3	**<.001**	0.70	0.70
	With Biochar	-0.03	0.01	16	-2.22	**0.041**		
	Drought	0.17	0.01	16	11.82	**<.001**		
	Invasive	-0.06	0.01	379	-4.85	**<.001**		
	With Biochar × drought	-0.26	0.02	16	-12.58	**<.001**		

H_{max} where invasive species grew larger in comparison to the natives (Fig. 3a–f, Table 2, and Suppl. material 1: table S1). Invasive species had higher values in comparison to native species for all the traits, except for LDMC (Fig. 3a–f). Even plants growing
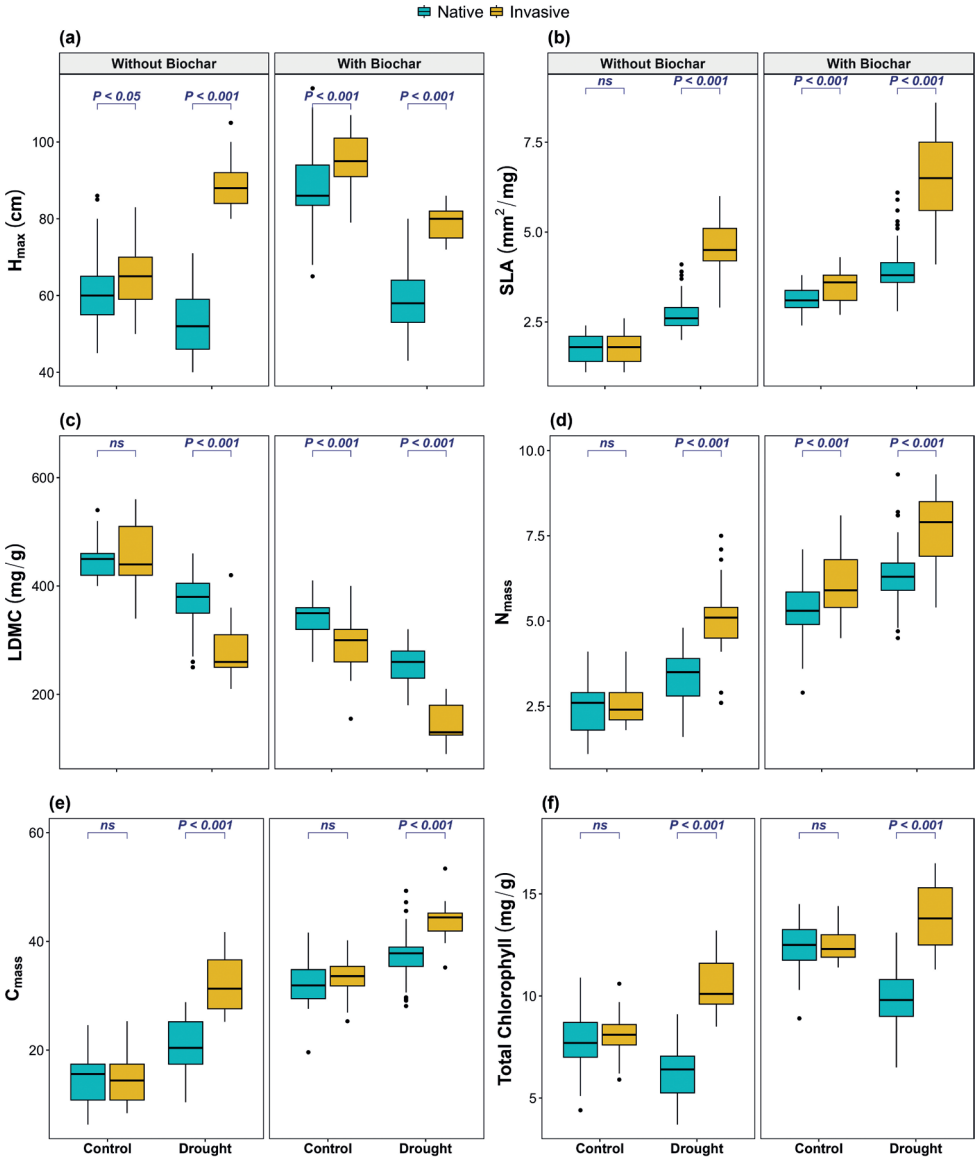
Figure 3. Effect of drought and Biochar application on (a) Maximum height (H_{max}), (b) Specific leaf area (SLA), (c) Leaf dry matter content (LDMC), (d) Leaf nitrogen content (N_{mass}), (e) leaf carbon content (C_{mass}), and (f) Total chlorophyll (Chl_{total}) of invasive and native species. Numbers are *P* values of the statistical significant differences between invasive and native species based on pairwise comparisons using Tukey's multiple comparison test (*ns*: non-significant differences).

with biochar showed significant differences between the native and invasive species, suggesting that the Biochar treatment favored the traits of the invasive species (higher H_{max}, SLA, and N_{mass}, and lower LDMC).

Effects of drought and Biochar on productivity

Our results showed that *A. fatua* had a significantly higher biomass in plots with drought in comparison to the three native species (Fig. 4a, Tables 2 and Suppl. material 1: fig. S1), these results were confirmed by the LMMs, as 82% of the variance was explained for total biomass (Table 2). The total biomass of the native species was increased with Biochar addition yet remained lower than the biomass of *A. fatua* (Fig. 4). The opposite trend was found in the RSR, as the native species had higher RSR than *A. fatua* under drought conditions (Fig. 4b, Table 2 and Suppl. material 1: fig. S1), the variance of the RSR were explained by the LMMs by 70% (Table 2) yet there was no significance difference between native and invasive species in terms of RSR under Biochar and control treatments (Fig. 4b).
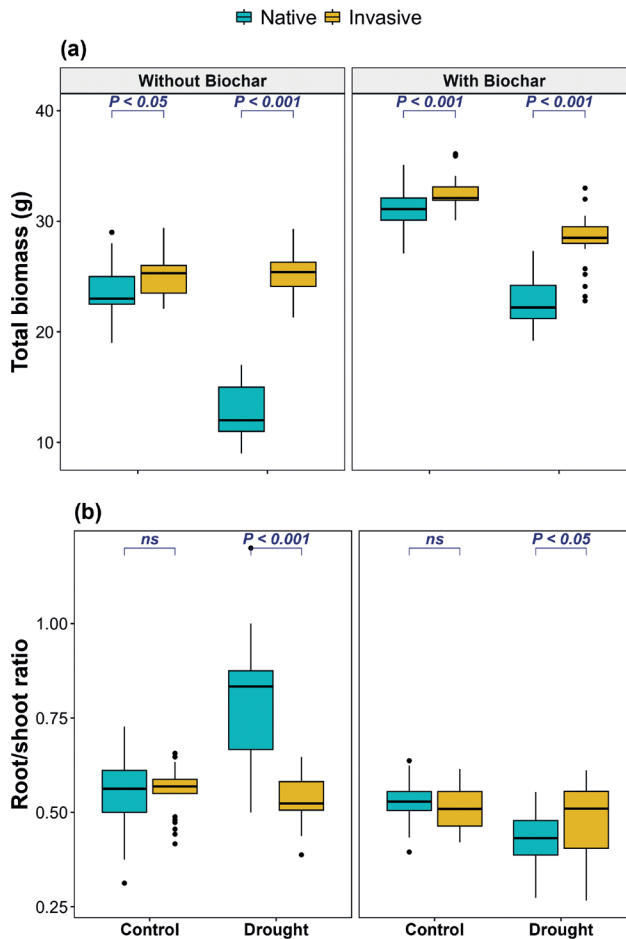

Figure 4. Effect of drought and Biochar application on (**a**) total biomass and (**b**) root to shoot ratio (RSR) of invasive and native species. Numbers are *P* values of the statistical significant differences between invasive and native species based on pairwise comparisons using Tukey's multiple comparison test (*ns*: non-significant differences).

Discussion

Under drought conditions Biochar application positively influenced both native and invasive species, especially under drought conditions, confirming previous studies which reported how Biochar can mitigate the adverse effects of drought conditions by improving soil physical, chemical and microbial content (Jien and Wang 2013; Hardy et al. 2019; Zheng et al. 2019; Sun et al. 2022). Based on function trait values there is an evidence for the superior competitive strength of the invasive species *Avena fatua* under drought condition which was enhanced by the addition of Biochar to have higher H_{max}, SLA, N_{mass} and Chl_{total} and lower LDMC, verifying that invasive species perform better than native species due to their superior traits related to resources acquisition (Allison and Vitousek 2004; Sardans et al. 2017). These findings were in contrast with previous research suggesting that using Biochar as soil amendment mitigate allelopathy produced by invasive species (Chen et al. 2022; Sujeeun and Thomas 2023; Xu et al. 2023). Finally, as *A. fatua* profited more from biochar application compared to native species, indicating that the overall performance of the species considered has improved, it is still likely that in the long term, *A. fatua* will take over with its larger SLA and higher leaf nitrogen, confirming its higher competitive strength.

Effect of drought and Biochar application on species performance of native and invasive species in artificial grassland communities

One of the important features of invasive species is their good performance that allows them to succeed and outcompete native species even under unfavorable conditions like drought. In the current study, we found significant differences in all the studied plant functional traits between invasive and native species under Biochar+drought and drought, confirming the high performance of the invasive species in comparison to the natives as also shown by (Funk et al. 2016; Mathakutha et al. 2019; Chen and van Kleunen 2022; Liu et al. 2022). Our findings on H_{max} are in line with previous studies, suggesting that under stress conditions (e.g., drought) H_{max} will be a vital measure and predictor of plant invasion (Grotkopp et al. 2002), assuming that tall plants have lower competition for resources (e.g., light) (Closset-Kopp et al. 2011) and consequently improved nutrient acquisition (Moles et al. 2009).

Higher SLA can reflect the efficiency of resource and nutrient acquisition (e.g., light and nitrogen) giving the invasive species advantage when compared to native species (Knops and Reinhart 2000; Gommers et al. 2013; Rosbakh et al. 2015). We found that *A. fatua* possessed higher SLA and lower LDMC under drought conditions with or without Biochar confirming the fast growth and high biomass production of invasive species in comparison with the native species (Hodgson et al. 2011) indicating the favorable resource use strategy of invasive species (Garnier et al. 2001). *A. fatua* has higher leaf N_{mass} when compared to the native species under drought and Biochar+drought conditions. This proved the ability of invasive species to capture more CO_2 within their leaves due to the effect of leaf nitrogen on improving leaf protein content, i.e., rubisco (Evans 1989; Wright et al. 2004). These findings confirm also the superiority

of invasive species in nutrient acquisition and improved photosynthetic rates in comparison with the native ones (Yang et al. 2020). Similarly, we found that under drought conditions plants accumulated more C_{mass} in comparison to the control conditions, which was proposed earlier as plants tend to maintain more C_{mass} under drought conditions to enhance leaf senescence (Sala et al. 2012; O'Brien et al. 2014; Hagedorn et al. 2016). Interestingly, invasive species *A. fatua* accumulated more C_{mass} in comparison to the native species suggesting that the invasive species got several strategies to efficiently use resources (Barros et al. 2020) making it more resistant to drought in comparison to the native species (Xing et al. 2021). *A. fatua* had a significantly higher Chl_{total} content than the native species under the two drought treatments. Such increase in Chl_{total} of *A. fatua* improved their capacity to harvest light under drought treatment in comparison to the native species (Zhuang et al. 2020) which consequently will lead to better photosynthetic capacity, and nutrient acquisition (Li et al. 2018a)

Effect of drought and Biochar application on species productivity of native and invasive species in artificial grassland communities

The present study found that the invasive species *A. fatua* had a significantly higher total biomass than the native species in both experimental plots. Previous studies also showed that under drought conditions, invasive species will have higher biomass production due to their strong plasticity (Funk et al. 2016; Ali and Bucher 2022). These findings also confirmed that *A. fatua* as an invasive species was more tolerant to drought conditions in comparison to native species as reported by Valliere et al. (2019). Moreover, invasive species exhibit traits that are linked to rapid growth and better resource acquisition in comparison to the native species (Leishman et al. 2007; van Kleunen et al. 2010; Dawson et al. 2012), that also make them more successful under changing climate. However, these effects were not as pronounced in the control treatment. Previous studies suggested that Biochar improves biomass production by improving soil chemical properties, e.g., soil pH, soil organic carbon content and C/N ratio (Zheng et al. 2019) as well as physical properties of soil, e.g., mean weight diameter of soil aggregates and thus reduce soil loss (Jien and Wang 2013; Sun et al. 2022) and help in improving soil microbial communities (Hardy et al. 2019).

Regarding RSR, native species showed significantly higher significant values than the invasive species under drought treatment, an opposite relation under Biochar+drought. These findings are a result of reduction in the aboveground biomass rather than an increase in root biomass, which confirmed previous findings that drought mainly affects aboveground biomass rather than the root biomass resulting in a strong allocation to roots to look for water (Lemoine et al. 2013). In a study by Mahajan and Tuteja (2005), leaves were more sensitive to drought conditions than roots. Finally, Biochar improved the RSR for native species as it increased the biomass production rather than affecting the root traits as reported by Xiang et al. (2017), where they showed that Biochar improved root length and the number of root tips more

strongly than on root diameter. One potential reason for the ability of Biochar to level out the differences between native and invasive species, is because Biochar can improve the soil pH, soil cation exchange capacity, and availability of several macronutrients, e.g., calcium, phosphorus, and potassium (Novak et al. 2009; Adams et al. 2013), which will make them more available for natives especially under drought conditions. Such improvement of soil properties due to the Biochar amendment was explained by several mechanisms, e.g., improved microbial activity and mycorrhizal-plant associations (Glaser and Amelung 2003; Drake et al. 2015; Gale et al. 2017).

Conclusions

In the present study, we clearly showed that drought did not have a negative impact on the invasive species *A. fatua*, which showed better overall trait conditions under drought. Overall, Biochar addition mitigated the negative effects of drought, but this mitigation favored the invasive species more than the native ones. Moreover, the performance of the invasive species was better than the native ones under drought conditions, which was clear in terms of plant functional traits (H_{max}, SLA, LDMC, N_{mass}, C_{mass}, and Chl_{total}). Based on the results of the current study, Biochar might be useful to mitigate climate change impacts, especially by fostering native species in Mediterranean grasslands unless not invaded by *A. fatua*. Moreover, using Biochar may be a useful tool for grassland restoration and conservation, especially under changing climate. As our conclusions were based on experimental plant communities, further studies focusing on long term effects of Biochar applications on more diverse and natural grasslands under field conditions are needed.

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Supplementary material 1

Supplementary information

Authors: Hamada E. Ali, Solveig Franziska Bucher, Markus Bernhardt-Römermann, Christine Römermann

Data type: doc

Explanation note: **figure S1.** Biochar application treatment for 10 plots as it received 1.25 kg of Biochar mixed with the topsoil before sowing. **figure S2.** PCA results of the plant functional responses (Maximum height (Hmax), Specific leaf area (SLA), Leaf dry matter content (LDMC), Leaf nitrogen content (Nmass), leaf carbon content (Cmass), Total chlorophyll (Chltotal)) and species production (total biomass and root to shoot ratio (RSR)) of native and invasive species as a response to the four different treatments (Biochar+drought, Biochar, drought, and control). **table S1.** Average amount of the plant functional responses (Maximum height (Hmax), Specific leaf area (SLA), Leaf dry matter content (LDMC), Leaf nitrogen content (Nmass), leaf carbon content (Cmass), Total chlorophyll (Chltotal)) and species production (total biomass and root to shoot ratio (RSR)) of native and invasive species as a response to the four different treatments (Biochar+drought, Biochar, drought, and control).

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