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Chelonian challenge: three alien species from North America are moving their reproductive boundaries in Central Europe

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Abstract

Biological invasions by alien species have substantial economic impacts and are a major driver of the ongoing decline and loss of biodiversity. Through humans, the North American pond slider (*Trachemys scripta*) has acquired a global distribution over the last decades and is currently listed among the worst invasive reptile species. However, in more recent times, other freshwater chelonian species have increasingly been recorded far outside their native distribution ranges as well, not only on the same continent but also on others. Despite that, the impact of alien chelonians on their respective new ecosystems remains unclear. The long-term effects and severity of impacts of alien populations mostly depend on whether they ultimately succeed in establishing themselves. This is not entirely resolved for chelonians in Central Europe. To answer that, we investigated wild populations of three non-native chelonian species from North America in Germany (*Pseudemys concinna*, *Graptemys pseudogeographica* and *Trachemys scripta*) applying population genetic approaches. We revealed the successful reproduction of all three species in Germany and provide the very first record for the reproduction of *P. concinna* and *G. pseudogeographica* in a temperate continental climate zone outside their native distribution. Based on our unambiguous evidence of natural reproduction, we call for dedicated studies to verify how widespread established populations are and to investigate the existing and potential impacts of all three species in a range of ecosystems along a climatic gradient. Such data is urgently needed to revise the current risk assessments of non-native chelonians, especially in Central European countries.

* These senior authors contributed equally to this work.

Keywords
biodiversity loss, biological invasion, continental climate, Europe, *Graptemys pseudogeographica*, population genetics, *Pseudemys concinna*, *Trachemys scripta*

Introduction

Biological invasions by alien species have substantial economic impacts (Essl et al. 2020; Diagne et al. 2021; Soto et al. 2022) and are also a major driver of the ongoing decline and loss of biodiversity (Butchart et al. 2010; Ripple et al. 2017; IPBES 2019a, b; Seebens et al. 2021). Despite that, the number of alien species is growing continuously (Pyšek et al. 2020). And regardless of an existing unifying framework for biological invasions (Blackburn et al. 2011), the terms "alien", "casual/introduced", "naturalised/established" and "invasive" are often not applied correctly in numerous scientific and non-scientific publications. This makes common language a challenge. In the following, for simplicity reasons, we consider a species alien if human actions enabled them to overcome biogeographical barriers and invasive once a population becomes established, e.g. exhibiting regular reproduction. Whether a species outside its native distributional range should be categorised as alien or invasive is our main underlying study question.

To highlight the serious impacts of invasive species, the IUCN Invasive Species Specialist Group's (ISSG) lists the "100 World's Worst Invasive Alien Species" (Lowe et al. 2000). It aims to illustrate a wide variety of examples from microorganisms, fungi, plants, invertebrates and vertebrates (Lowe et al. 2000). At first glance, it seems paradoxical that some of the taxa listed therein are also the world's most threatened ones. For example, chelonians (Reptilia: Order Testudines) are among the most imperiled vertebrates on the planet (Lovich et al. 2018; Rhodin et al. 2018; Stanford et al. 2018; Cox et al. 2022) with over 60% of the species listed as threatened by extinction (IUCN 2021; Cox et al. 2022). At the same time the pond slider *Trachemys scripta* (Thunberg in Schoepff, 1792) is listed among the worst invasive reptile species (Lowe et al. 2000). Furthermore, at least three other freshwater chelonians have a substantial risk of becoming invasive (Bugter et al. 2011).

Trachemys scripta is native to south-eastern North America (Ernst and Lovich 2009; Vamberger et al. 2020) and meanwhile has acquired a global distribution, being widespread on all continents except Antarctica (Kikillus et al. 2010; Uetz et al. 2022). Due to their popularity as pets, especially the subspecies *T. scripta elegans*, they were massively imported to Europe in the 1980s and 1990s (Arvy and Servan 1998; Ernst and Lovich 2009; Vamberger et al. 2012) and released in water bodies. In 1997, the European Union banned imports of *T. scripta elegans* (Commission Regulation EC 338/1997). Sales of individuals born in EU member states were not forbidden until 2016 (EU Regulation 1143/2014 on Invasive Alien Species), which then included all

subspecies of *T. scripta*. However, by then *T. scripta* was already widely present in water bodies all over Europe (Kikillus et al. 2010; Standfuss et al. 2016; Uetz et al. 2022 and references therein). Unfortunately, these regulations seemed to have caused a shift in demand for freshwater chelonians. Two other subspecies, *T. scripta scripta* and *T. scripta troostii*, as well as several other species, especially of the genera *Pelodiscus*, *Pseudemys*, *Graptemys*, and *Chrysemys*, have replaced *T. scripta elegans* in the pet trade (Rhodin et al. 2010; Bugter et al. 2011; Carretero and Pinya 2011; Lipovšek 2013; Brejcha et al. 2014; Escoriza et al. 2021; Uetz et al. 2022 and references therein) and are also illegally released into numerous freshwater bodies around the world.

The impacts of these alien chelonians on their respective ecosystems remain largely unclear (see also Bugter et al. 2011). So far, studies have focused on direct impacts on other chelonians, e.g. the European pond terrapin *Emys orbicularis* (Cadi and Joly 2004), which is of conservation concern in many countries in Europe. Despite the proximal causes remaining unknown, in an experimental setup native *E. orbicularis* showed weight loss and high mortality when kept together with *T. scripta*. The most likely suggestion seems that the larger alien species exclude the smaller native ones from basking spots and thus the latter suffer from suboptimal thermoregulation (Cadi and Joly 2004). There are also hints that native amphibian larvae recognise native freshwater chelonians as predators but not alien ones (Polo-Cavia et al. 2010) so alien *T. scripta* might have feeding advantages. Other effects are not studied but experimental evidence suggests a key role of chelonians in ecosystem functioning, altering, for example, sediment accumulation, leaf litter decomposition rates and abundance of invertebrates (Lindsay et al. 2013; Dupuis-Desormeaux et al. 2022). This indicates potentially severe impacts outside their native ranges. However, before studying impacts we must consider the question of whether alien populations are established, i.e. whether they are regularly reproducing in the wild outside their native range (Bugter et al. 2011).

We investigated wild populations of three non-native chelonian species (river cooters *Pseudemys concinna*, false map turtles *Graptemys pseudogeographica* and pond sliders *Trachemys scripta*) using population genetic approaches with 14 microsatellite loci and performing parentage analyses. Our assumptions are that reproduction in the wild occurs, if (i) juveniles are found in the wild, (ii) closely related individuals are recorded and (iii) that a population has established itself when at least half of the studied markers are in Hardy–Weinberg equilibrium (HWE) (following Standfuss et al. 2016). HWE is reached, when allele and genotype frequencies in a population remain constant from generation to generation, thus there is an absence of other influences on the population (e.g. immigration). However, HWE cannot be achieved if continuously new alleles are added to a population, in other words through continuous releases of non-native chelonians. In addition, detection of unrelated individuals would suggest repeated releases of chelonians and no reproduction in their exotic environments. Herein we unravel whether these three species have formed self-sustaining populations in south-western Germany outside their native distribution ranges, which would be the first time for *P. concinna* and *G. pseudogeographica* and the most northern record for *T. scripta*.

Methods

Study sites

Based on informal reports of relatively large populations of pond sliders *Trachemys scripta*, in situ inspections of several water bodies, and observations of hatchlings of *T. scripta* in Kehl (Pieh and Laufer 2006; Schradin 2020), two study sites in Germany were selected (Fig. 1; Suppl. material 1: fig. S1). Our first study site, "Flückigersee" (48°00'38"N, 7°49'06"E, abbreviated FR) is a dredging lake located in the middle of the city of Freiburg im Breisgau, categorised as a semi-natural lake with a

Figure 1. Map of Baden-Württemberg, Germany, with locations and satellite photos of both study sites. Hill shade symbolises elevation, forest cover is illustrated in green and urban areas in light red. The main map shows the location of the sites within Baden-Württemberg and top left within Germany. Map was created with QGIS (QGIS Development Team 2020).

size of 11.2 ha, and maximum depth of 26.8 m (LUBW 2020) (Fig. 1). Its elevation is 240 m a.s.l., annual mean temperature in Freiburg is 10.3 °C (Climate-Data.org 2020). The second study site "Altrhein" (Fig. 1; Suppl. material 1: fig. S1) is a 3.3 ha standing oxbow lake located in the city of Kehl (48°34'04"N, 7°48'37"E, abbreviated KE), disconnected from the current course of the river Rhine (LUBW 2020). Its elevation is 139 m a.s.l. and the city's annual mean temperature is 11.1 °C (Climate-Data.org 2020). Both water bodies are located in urban parks and are completely surrounded by residential areas.

Fieldwork

Fieldwork was conducted between May and August 2020. We caught chelonians opportunistically by hand, dip netting, a non-baited basking trap and ten baited funnel-traps with modified elastic entrances to enable large individuals to enter the traps. Funnel-traps were baited with chicken heart, chicken liver, beef or mixtures of anchovies, mackerel, codfish liver and cat food. They were placed in shallow areas, tied to nearby vegetation, using buoys to ensure that the traps could not submerge completely.

Captured living chelonians had blood drawn from the sub-carapacial space above the neck for genetic analyses. Tissue samples were taken only from dead individuals. We used Whatman FTA Cards (GE Healthcare Life Sciences, Chalfont St Giles, GB) and ethanol for preservation of blood samples. Sex was determined for individuals above 9 cm carapax length, using secondary sexual characteristics such as elongated claws on forelimbs (only present in males) and position of the cloacal opening (in females closer to the shell then in males) (Ernst and Lovich 2009). Age was estimated as a combination of the number of growth rings and shell abrasions (Govedič et al. 2020). Juveniles represent individuals of up to the age of 2 years (2 growth rings). Based on shell abrasion, adults were divided into three classes: young adults, middle-aged adults and old adults (Meeske 2006; Vamberger and Kos 2011; see Table 1). All aspects of field work were approved under permit number 35-9185.81/G-20/06 by the "Regierungspräsidium Freiburg, Abteilung 3" of the German federal state of Baden-Württemberg.

DNA extraction, PCR and microsatellites

We extracted genomic DNA from FTA cards by using the illustra Tissue and Cells genomicPrep Mini Spin Kit (GE Healthcare Life Sciences). For extraction of DNA from blood, tissue and cloaca swabs preserved in ethanol we used the innuPREP Blood DNA Mini Kit (Analytik Jena GmbH). For amplification of microsatellite DNA, three Multiplex-PCRs (MP 1–3; Suppl. material 1: table S1) were performed using the Qiagen Type-it Microsatellite PCR Kit (QIAGEN GmbH). Thermocycling conditions were as follows: one cycle of initial denaturation (95 °C; 5 min), 30 cycles of denaturation (95 °C; 30 sec), annealing (55 °C; 90 sec) and elongation (72 °C; 30 sec) and one cycle of final elongation (60 °C; 30 min). For reaction mixes we followed the protocol of Standfuss et al. (2016). Fragment length analysis was conducted on an ABI 3130xl

Table 1. Number of chelonians caught and analysed genetically, sorted by population and split by age classes and sex for all three species analysed. Sex determination of hatchlings and subadults is not possible, due to the absence of distinct sexual characters.

Population	Total	Adult females	Adult males	Sub	Ha
P. c. (FR)	33	21 (14 OA, 5 MA, 2 YA)	3 (2 OA, 0 MA, 1 YA)	3	6
G. p. (FR)	25	11 (8 OA, 3 MA, 0 YA)	5 (1 OA, 1 MA, 3 YA)	6	3
T. s. (FR)	71	35 (14 OA, 15 MA, 6 YA)	12 (5 OA, 4 MA, 3 YA)	20	4
P. c. (KE)	2	0	0	2	0
T. s. (KE)	56	21 (7 OA, 6 MA, 8 YA)	8 (2 OA, 2 MA, 4YA)	21	6

P. c. = *Pseudemys concinna*, *G. p.* = *Graptemys pseudogeographica*, *T. s.* = *Trachemys scripta*, FR = Freiburg, KE = Kehl, Sub = subadult, Ha = hatchling, OA = old adults, MA = middle-aged adults, YA = young adults; see methods for further details.

Genetic Analyzer (Life Technologies). For final determination of fragment lengths, we used the software PEAK SCANNER 1.0 (Applied Biosystems). Errors in genotyping were minimised by re-amplification of samples that showed weak or missing signals.

Cross-amplification tests of microsatellites

First we tested the applicability of the 14 microsatellite loci (Suppl. material 1: table S1) for one individual per species of *Pseudemys concinna* and *Graptemys pseudogeographica* as these were originally developed for *Trachemys scripta* (Simison et al. 2013) through cross-amplification tests. For PCR reaction we used a primer concentration of 0.025 mM (Biomers.net, Ulm, Germany) and otherwise followed the protocol of Standfuss et al. (2016).

PCRs were conducted under thermocycling conditions provided in Standfuss et al. (2016). In case of amplification, the presence of the microsatellites was confirmed by sequencing the PCR products with primers in both directions. PCR products were purified with the ExoSAP-IT enzymatic cleanup (Thermofisher, Waltham, USA) and sequenced using the reverse primer of each locus on an ABI 3130xl using the Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The applicability of each microsatellite loci on *Pseudemys concinna* and *Graptemys pseudogeographica* was approved by confirming the presence of the expected repeat motives, checked with BIOEDIT (Hall 2011).

Genetic diversity indices and cluster analysis

We used CONVERT 1.31 (Glaubitz 2004), PEAK SCANNER 1.0 (Applied Biosystems), CERVUS 3.0 (Kalinowski et al. 2007) and ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010) to analyse microsatellite data (AMOVA) and calculate genetic diversity indices for all three species. Genetic cluster analyses were performed for *T. scripta* from FR and KE using an unsupervised Bayesian clustering approach, implemented in the software STRUCTURE 2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009) to analyse whether the two populations correspond to separate clusters and form established and breeding populations. STRUCTURE searches for populations in Hardy-Weinberg

equilibrium (HWE) and linkage equilibrium. In the analyses we applied the admixture model and correlated allele frequencies and set the upper bound for calculations arbitrarily to $K = 10$. Although we sampled two populations, the upper bound was set higher to exclude potential source populations from which the animals could be released. Because MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) suggested the presence of null alleles (Suppl. material 1: table S2), data were corrected for null alleles according to Falush et al. (2007). The most likely number of populations (K) was determined by using the Ln P(D) (mean likelihood of K) values (Pritchard et al. 2000) and the ΔK method (Evanno et al. 2005), implemented in the software STRUCTURE HARVESTER (Earl and vonHoldt 2012). We repeated calculations 10 times for each K using a MCMC chain of 750 000 generations for each run, including a burn-in of 250 000 generations. Population structuring and individual admixture were visualised using the software DISTRUCT 1.1 (Rosenberg 2004). Following Randi (2008), we categorised individuals with proportions of cluster membership below 80% as having mixed ancestries.

Kinship analysis

We calculated the most likely relationships between individuals of each species at each study site. Therefore, we applied a maximum likelihood approach for pairwise estimates of relatedness and computed Wright's coefficient (r) of relatedness, implemented in ML-RELATE (Kalinowski et al. 2006). Null alleles detected by MICRO-CHECKER were accommodated by ML-RELATE. Kinship analyses were conducted for respective populations of *Pseudemys concinna*, *Graptemys pseudogeographica* and *Trachemys scripta*, from FR and one population of *Trachemys scripta* and two individuals of *Pseudemys concinna* from KE. Analyses were conducted for all relationships available in the software (U = unrelated, HS = half sibling, FS = full sibling and PO = parent-offspring). Confidence level for estimated relationships in ML-RELATE was set at 95% by running 100 000 simulations. When ML-RELATE suggested more than one relationship, we executed a specific hypothesis test for two a priori relationships by means of a likelihood ratio test. We checked all genetically identified relationships against our morphological data (e.g. age class; see Table 1) for potential errors (such as offspring older than presumed parent); no such erroneous classification was observed.

Results

In total, we sampled 33 individuals of *Pseudemys concinna*, 25 of *Graptemys pseudogeographica* and 71 of *Trachemys scripta* from FR and 56 *Trachemys scripta* and 2 *Pseudemys concinna* from KE (for more details see Table 1). Subspecies of *T. scripta* were assigned as follows: FR: 42 *T. s. elega*ns, 16 *T. s. scripta*, 9 *T. s. elegans* × *scripta* hybrids and 4 *T. scripta* which could not be assigned to a subspecies or hybrid; KE: 37 *T. s. elegans*, 6 *T. s. scripta*, 8 *T. s. elegans* × *scripta* hybrids and 5 *T. scripta* which could not be assigned to a subspecies or hybrid.

Cross-amplification tests of microsatellites

Out of 14 microsatellite loci, originally developed for *T. scripta* (Suppl. material 1: table S3), only one failed to amplify (Tsc297) in *P. concinna*, while all amplified for *G. pseudogeographica.* All analysed microsatellite loci turned out to be polymorphic for *G. pseudogeographica* (Suppl. material 1: tables S2, S3) while for *P. concinna* locus Tsc108 was monomorphic (Suppl. material 1: table S3). Accordingly, loci Tsc297 and Tsc108 were excluded from further analyses for *P. concinna*.

Genetic diversity indices

The highest average number of alleles per locus ($A_{Ø}$) was revealed in *T. scripta* FR (15.1) followed by *T. scripta* KE (13.1), *P. concinna* FR (9.1) and *G. pseudogeographica* FR (9) (Table 2). MICROCHECKER detected null alleles in all analysed species (Table 2). Numbers of private alleles ranged from a minimum of 12 in *G. pseudogeographica* (FR) to a maximum of 23 in *T. scripta* (FR). More than half of the loci were in Hardy-Weinberg equilibrium (HWE) in *P. concinna* (FR: 8/12), *G. pseudogeographica* (FR: 7/14) and *T. scripta* (FR: 9/14), while only four out of 14 were in HWE in *T. scripta* in KE (Table 2).

Genetic cluster analysis

Using all 14 microsatellite loci and the correction for null alleles in STRUCTURE, we examined whether *T. scripta* from each site (FR and KE) correspond to a population in Hardy–Weinberg and linkage equilibrium. Ln P(D) values and the Δ*K* method suggested $K = 2$ being the most likely number of clusters (Suppl. material 1: fig. S2), each corresponding to the respective study site (Fig. 2). However, several individuals from KE (yellow cluster; Fig. 2) clustered within corresponding individuals from FR (red cluster Fig. 2). A standard AMOVA revealed a statistically significant molecular difference between the two populations of *T. scripta* (FR and KE) of 2.01% (Fst-value: 0.02; $p < 0.001$).

Table 2. Genetic diversity indicators of all four chelonian populations, based on 12 microsatellite loci for *P. concinna* and 14 microsatellite loci for *G. pseudogeographica* and *T. scripta*.

Population	A_N	$A_Ø$	A_0	A_p	$H_{EØ}$	$H_{OØ}$	HWE_N	*PO*	*FS*	*HS*
P. concinna (FR)	109	9.1	2	20	0.70	0.75	8	7	14	48
G. pseudogeographica (FR)	126	9	3	12	0.72	0.79	7	2	18	32
T. scripta (FR)	212	15.1	4	23	0.76	0.88	9	12	18	154
T. scripta (KE)	184	13.1	5	13	0.77	0.86	4	7	49	125

FR Freiburg, ***KE*** Kehl, A_N number of alleles, $A_Ø$ average number of alleles, A_0 number of loci with null alleles, A_p private alleles, $H_{EØ}$ average of expected heterozygosity, $H_{OØ}$ average of observed heterozygosity, HWE_N number of loci in Hardy-Weinberg equilibrium, ***PO*** number of parent-offspring-relationships, ***FS*** number of full-sibling -relationships, ***HS*** number of half-sibling relationships.

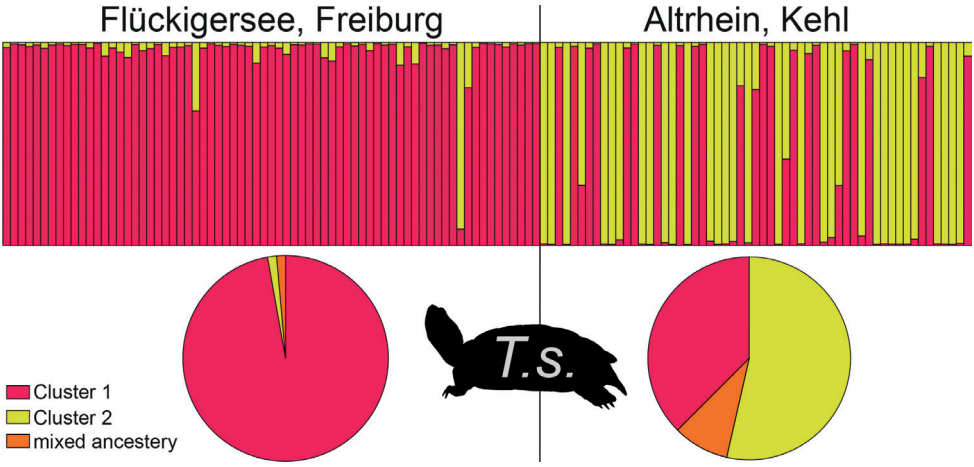

Figure 2. Population structuring in *Trachemys scripta* (*T.s.*) for $K = 2$ from the STRUCTURE run with the highest probability value. Revealed cluster (red, yellow) are presented in distinct colours. Each vertical bar represents one individual and its calculated proportion of cluster membership. Colours of pie charts correspond to STRUCTURE clusters; orange slices represent chelonians with mixed ancestry (percentages).

Kinship analysis

We detected the full variety of kinship relationships within all analysed populations. Parent-offspring-relationships were confirmed for all three species (Table 2, Suppl. material 1: tables S4–S7). Analysis of *P. concinna* from FR revealed kinship relationships among 28 of 34 (82%) sampled individuals (Suppl. material 1: table S4). The two analysed subadult individuals from KE showed a full-sibling-relationship; so far no hatchlings of *P. concinna* have been detected in KE. Kinship relationships were revealed for 18 of 25 (72%) sampled *G. pseudogeographica* (Suppl. material 1: table S5). In *T. scripta*, 58 of 71 (82%) analysed individuals from FR (Suppl. material 1: table S6) and 45 of 57 (79%) from KE (Suppl. material 1: table S7) showed kinship relations with at least one other individual. We genetically confirmed hybridisation between two subspecies of *T. scripta* in FR for one hatchling (no. 22367), detecting a parent-offspring-relationship for a male *T. s. elegans* (no. 22300) and a female *T. s. scripta* (no. 22248) (Suppl. material 1: table S6). Correspondingly, this hatchling exhibited intermediate morphological characters of both subspecies (see Suppl. material 1: fig. S3).

Discussion

For the first time we genetically confirm successful reproduction of three alien chelonian species in Germany. For two species, *Pseudemys conncina* and *Graptemys pseudogeographica*, reproduction in Germany (FR) is the first record in a temperate

continental region and for each species it is the second outside their native distribution range in North America (see below). The detected full-sibling-relationship between the two analysed subadult individuals from KE does not prove reproduction in KE, but it can be suggested. This is in line with our capture data, and so far we have not detected hatchlings of *P. concinna* in KE. Both species are popular in the European pet trade and are occasionally noted as alien species in the wild (Ottonello et al. 2014; Foglini and Salvi 2017; Ferri et al. 2020; Uetz et al. 2022 and references therein).

For *Trachemys scripta*, our genetic confirmation of suspected natural reproduction in FR and KE are the first ones for Germany, the northernmost for the species in Europe to date and the second one for a temperate continental region. Successful reproduction and self-sustaining populations of *Trachemys scripta* in Europe were previously known from Mediterranean regions (e.g. Cadi et al. 2004; Perez-Santigosa et al. 2008; Ficetola et al. 2012; Sancho and Lacomba 2016; Vecchioni et al. accepted) and the temperate continental climatic zone of Slovenia (Standfuss et al. 2016). In Germany, hatchlings of *T. scripta* have been previously sighted and documented in KE (Pieh and Laufer 2006; Schradin 2020). Egg laying (without observations of hatching events) as well as increasing numbers of juveniles were reported by Schradin (2020). Thus Pieh and Laufer (2006) and Schradin (2020) concluded that successful reproduction occurs. For *P. conncina* behavioural observations and a single juvenile in an animal shelter suggested successful reproduction under semi-natural conditions in Catalonia in Spain (Soler and Martínez-Silvestre 2020). For *G. pseudogeographica* two nesting events with partly successful hatching events in urban parks in Brescia and Milano, Italy, were observed but not published (Ferri et al. 2021).

Herewith, our data confirms these previous assumptions. Not only do we provide evidence for reproduction of three species of alien chelonians, derived from parentage analyses confirming numerous relationships (parent-offspring, full-sibling and half-sibling) between individuals (Suppl. material 1: tables S4–S7), our calculated genetic diversity indices reveal HWE for the majority of markers (Table 2; Suppl. material 1: tables S2, S3). The sampled populations seem to represent established populations, meaning they are populations with no external influences on population growth and age composition, i.e. no releases of new individuals.

For *T. scripta*, this assumption is supported by the similarity of diversity indices between both sites, especially the average heterozygosity (*T. scripta* FR: 0.88; KE: 0.86 in our study) compared to the native populations (e.g. 0.81 in Simison et al. 2013). Even though sample sizes of *P. concinna* and *G. pseudogeographica* were lower than for *T. scripta*, the numbers of loci in Hardy-Weinberg-Equilibrium are similar among all three species in FR (Table 2) and similar to populations of *T. scripta* in Slovenia (Standfuss et al. 2016). With the exception of *T. scripta* in KE, the observed high numbers of loci in HWE are remarkable, considering the anthropogenic origin of the studied populations. In general, chelonians mature sexually relatively late, especially females, between 8 to 12 years in *G. pseudogeographica* (3–4 years in males), 13 to 24 years for *P. concinna* (6 years in males) and 6 to 8 years in *T. scripta* (2–5 years in males) (Ernst and Lovich 2009). The continuous release of non-native chelonians counteracts the potential to achieve complete HWE (Standfuss et al. 2016). Nevertheless, our STRUCTURE analysis of

T. scripta populations from FR and KE revealed two most probable clusters, each corresponding to one sampling site. The population in FR seems to be already longer established than the one in KE, which is indicated by its genotypic structure (Fig. 2) and number of loci in HWE (Table 2) but no direct observational data is available.

In KE *T. scripta* was very common in the Altrhein until 2004 when the water body was restored and the population declined (Pieh and Laufer 2006). Only a single juvenile *T. scripta elegans* was found in 2004 (Pieh and Laufer 2006). During our fieldwork in 2020 we instantly detected numerous juveniles (also hatchlings) at basking sites and caught seven hatchlings and 18 sub-adults in 8 days. Evidently, the population in Kehl has increased within the last approximately 15 years, indicating that it is relatively young (see also Schradin 2020).

For successful establishment of invasive species, a viable sex ratio is important. Our data (see Table 1) indicates a sex bias towards females in adult chelonians but we believe, without having data to support our view, that males in our study system, especially in *T scripta*, are more difficult to catch than females. As males can mate with several females we do not believe that the observed sex ratio is a limiting factor and a female bias seems to be common in chelonians (Ewert and Nelson 1991; Ewert et al. 2004). Although we had no means of verifying the sex of subadult or hatchling chelonians, young adult males were caught in all three species. All three species exhibit "temperature dependent sex determination" (TSD) and therefore there could be a link between establishment at new localities and viable sex ratios. TSD is quite complex and relatively little studied across a large number of chelonian species. Changing climatic conditions, either through global change or relocation of species into novel environments, do not necessarily relate to different sex ratios in offspring. This is because molecular mechanisms, that are still being untangled, may play a significant role. A candidate gene for sex determination in *T. scripta* has previously been suggested (Ge et al. 2018). Environmental proxies influencing TSD in chelonians can be mean, range and variance of temperature at specific time periods (Girondot et al. 2010). In addition, ambient temperature is the most crucial factor but rarely known (Cornejo-Páramo et al. 2020). In contrast, there are publications showing that species with TSD can adapt to different climates, for example behaviourally through varying depths at which eggs are laid, locations of nest sites and timing of egg laying (Ewert et al. 2005; Schwanz and Janzen 2008; Refsnider and Janzen 2012; Pike 2013). Also embryos seem to have the possibility to thermoregulate inside their eggs (Shine and Du 2018). Another important point is that most models estimating pivotal temperatures, i.e. thresholds above or below only one sex is produced, still have to be validated through field data because they generally assume constant temperatures during the whole incubation period (e.g. Girondot 1999; Godfrey et al. 2003; Hulin et al. 2009) which is not necessarily always the case. Even if single clutches produce predominantly one sex, population dynamics of TSD are not well known (Ewert and Nelson 1991; Ewert et al. 2005) and it is unclear to our knowledge how that translates into viable sex ratios. There is even the suggestion that highly variable environments during development of embryos could facilitate adaptation at later life stages (Jonsson et al. 2022). So novel climates and TSD alone do not necessarily lead to sex biases and biases in viable

sex ratios. More field measurements at clutch sites across a range of climatic zones are required to reveal if, and how, viable sex ratios are achieved.

Overall, our results demonstrate the ability of three alien chelonian species to reproduce and establish viable populations in two sites in the Upper Rhine Plain in Baden-Württemberg, south-western Germany, which is considered to be one of the warmest regions in Germany. Both sites are urban habitats but alien chelonians are found in a large variety of water bodies, ranging from urban to natural, even within protected areas (Turtle Spotter 2020; pers. obs.). Therefore, it needs to be assessed whether unnoticed reproduction might have occurred also in other regions, in other alien species and more natural habitat types. For example, two hatchlings of *Chrysemys picta bellii* were observed near Speyer, Germany (Fritz and Lehmann 2002) and egg depositions of *T. scripta* were recently observed in more northern regions of Germany, e.g. Essen/Ruhr (Rautenberg and Schlüpmann 2018) and Saarbrücken (Francke pers. comm.). This is especially important in habitats where endangered species could be affected. Direct effects are potential competition with native chelonians. So far, observations indicate that *T. scripta* has competitive superiority to the endangered *E. orbicularis*, e.g. in procuring food (Nishizawa et al. 2014) and basking behaviour (Cadi and Joly 2004; Polo-Cavia et al. 2010, 2015). It was also demonstrated that *T. scripta* has physiological advantages, like faster chemosensory responses and superior thermoregulation (Polo-Cavia et al. 2008, 2009, 2012). Therefore, the abundance of alien chelonians might threaten the survival of endangered native populations of *E. orbicularis* in north-eastern Germany (Brandenburg) which harbours the last relict populations (Schneeweiß and Wolf 2009) but also in Europe in general. In addition, ongoing successful reintroduction programmes of *E. orbicularis* (Fritz and Chiari 2013) can be jeopardised by alien chelonians. In Germany, reintroduction efforts of *E. orbicularis* have been made in the federal states of Hessen and Rheinland-Pfalz (Fritz and Chiari 2013), in comparable latitudes where egg depositions of *T. scripta* were observed (Rautenberg and Schlüpmann 2018, Francke pers. comm.).

The almost omnivorous feeding behaviours of many alien chelonians might also have direct effects, indicating that rare and threatened native species of flora and fauna could by preyed upon. However, so far no data regarding food items of alien chelonians in central Europe are available. Besides these direct effects, a number of indirect effects are plausible and gaining research attention in recent times. For example, there is the risk of alien chelonians introducing and acting as reservoirs for novel diseases and parasites (Shen et al. 2011; Gong et al. 2014; Héritier et al. 2017). *T. scripta* is a known carrier of several salmonella and pathogens (Shen et al. 2011; Gong et al. 2014) and is also vulnerable to ranavirus (Moore et al. 2014). Cases of parasite transfer (e.g. helminths) from alien to native chelonians are already known from Europe (Héritier et al. 2017). Data about indirect impacts of other alien chelonians in Europe, besides *T. scripta*, are missing completely. In addition to negative effects, it was recently argued that alien chelonians in general, and *T. scripta elegans* in particular, might have the potential to offer ecosystem services in degraded ecosystems which would otherwise be lacking (Dupuis-Desormeaux et al. 2022). Thus, a differentiated view on alien chelonians in a range of habitats is called for.

Currently, the legislative restrictions for the pet trade, for example within the European Union, constrains only *T. scripta*. The other two species, *P. concinna* and *G. pseudogeographica*, are not included, and thus they are legally imported and available. It has to be assessed how widespread these species are in ecosystems outside their native range and whether control of the legal trade is necessary. Nevertheless, the focus should be laid on developing a diverse set of large public outreach campaigns to raise awareness of potential harmful impacts of releasing pets into the wild, for both the pet and the ecosystem it is released into (Teillac-Deschamps et al. 2009; Masin et al. 2014). Another step in a similar vein would be the further establishment of a "certificate of competence" (German: "Sachkundenachweis") for keeping exotic pets. During such a course potential keepers learn what resources are required to care for these animals adequately and the harm caused by illegal releases. This is already successfully established for aquarium and terrarium hobbyists in Germany (https://www.sachkunde-vda-dght.de/) and could become part of a general legal requirement. Education and public outreach can be accompanied by measures such as obligatory PIT tags (passive integrated transponder) for chelonians which can allow identification of their origin if found in the wild, thereby allowing the prosecution of illegal releases. Furthermore, a "conservation fee" could be charged when selling chelonians. Such a fee would increase costs for the keepers, hopefully lead to more careful considerations before purchasing these animals and it could be used to finance further research and fund rescue centres for released chelonians. In general, a more coordinated and positive approach to keeping exotic pets has numerous positive side effects (for example, see the recent efforts by Citizen Conservation https://citizen-conservation.org/?lang=en) such as raising awareness about the ongoing biodiversity crisis, emphasising responsible keepers and at the same time saving threatened species from extinction.

In conclusion, our results provide evidence for the novel establishment of four populations of alien chelonians belonging to three species in a temperate climate zone and thereby confirming earlier risk assessments (see Bugter et al. 2011). Remarkably, even *G. pseudogeographica* which is sensitive to cold (Ernst and Lovich 2009) reproduces in Germany and indicates that the risk assessment of *T. scripta* and other non-native chelonians species (see Bugter et al. 2011; Masin et al. 2014) should be generally revised based on scientific evidence, professional long-term monitoring efforts and if necessary adjusted accordingly, especially in Central European countries.

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Supplementary material 1

Supplementary information

Authors: Benno Tietz, Johannes Penner, Melita Vamberger

Data type: tables and figures (pdf file)

Explanation note: ID, multiplex-PCR numbers, annealing temperatures, repeat motifs, fragment sizes, fluorescent labelling of all used microsatellite loci. Genetic diversity indices of *Trachemys scripta* populations of Freiburg and Kehl. Genetic diversity indices of *Pseudemys concinna* and *Graptemys pseudogeographica*. Kinship-relationship-matrix of *Pseudemys concinna* individuals, generated by ML-RELATE. Kinship-relationship-matrix for *Graptemys pseudogeographica* individuals from Freiburg, generated by ML-RELATE. Kinship-relationship-matrix of *Trachemys scripta* individuals from Freiburg, generated by ML-RELATE. Kinship-relationship-matrix of *Trachemys scripta* individuals from Kehl, generated by ML-RELATE. Impressions of both habitats plus pictures of each species and the respective juveniles caught. Estimated log probability of data — Ln P(D), the mean likelihood of K (Delta K) and the number of simulated clusters for both *T. scripta* populations. Head and neck pattern in combination with plastral pattern of the hatchling 22367, showing intermediate morphological characters of both *Trachemys scripta* subspecies *Trachemys scripta elegans* and *Trachemys scripta scripta*.

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The TOP-100 most dangerous invasive alien species in Northern Eurasia: invasion trends and species distribution modelling

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Abstract

Northern Eurasia is extensive and includes terrestrial and aquatic ecosystems that cover several natural zones and access to the seas of three oceans. As a result, it has been invaded by numerous invasive alien species (IAS) over large temporal and spatial scales. The purpose of this research was to assess invasion trends and construct species distribution models for the Russian TOP-100 most dangerous IAS. Environmentally suitable regions for IAS were established based on alien species attribute databases, datasets of 169,709 species occurrence records (SOR) and raster layers of environmental variables using species distribution modelling (MaxEnt). The objectives of this research were to (1) create databases of SOR for the TOP-100 IAS in Russia; 2) determine pathways, residence time, donor regions and trends of invasions; (3) determine the main types of spatial distributions of invasive species and their relation to residence time; and (4) distinguish regions with the highest richness of IAS that have a strong impact on the terrestrial and aquatic ecosystems of Russia. We found that although species invasions date back over 400 years, the number of naturalized IAS has increased non-linearly over the past 76 years. The TOP-100 list is mainly represented by unintentionally introduced species (62%) which are characterized by different introduction pathways. Species occurrence records revealed that 56 IAS are distributed locally, 26 are distributed regionally and 18 are widespread in Russia. Species with local, regional or widespread distributions were characterized by residence times of 55, 126 or 190 years, respectively. We found that IAS with local distribution can expand their range into suitable regions more extensively (expected increase by 32%) than widespread species (expected increase by only 7%). The procedure of identifying hot/cold spots locations based on SOR allowed us to identify the Russian regions with the highest richness of IAS. Our results and the integrated database that we created provide a framework for studying IAS over large temporal and spatial scales that can be used in the development of management plans for dangerous IAS.

Keywords
animals, biological invasions, donor regions, hot spots, microorganisms, plants, SDM, species occurrence records

Introduction

Globalization, changes in climate and land use, and increased traffic flows have accelerated the rates of introduction of invasive alien species (IAS) to unprecedented levels and allowed them to overcome fundamental biogeographic barriers (Richardson 2011; Seebens et al. 2018). This increase in the spread of IAS is one of the principal features of the Anthropocene Era (Crutzen 2002; Lewis and Maslin 2015). Many countries around the world develop and implement science-based biosafety strategies in response to current and potential invasions of alien species (Wittenberg and Cock 2001; Clout and Williams 2009) to conserve biodiversity (Convention on Biological Diversity 2010) and promote the sustainable development of ecosystems (Transforming our world 2015). One of the challenges for minimizing the risk of invasions and potential losses in the environmental, socio-economic and medical spheres at local, regional and global levels is to identify priority alien species and locations to incorporate into ecological management (Wittenberg and Cock 2001; Stohlgren and Jarnevich 2009). As part of such activity, the 100 most dangerous IAS for the World (TOP-100 World) were identified (Weijden et al. 2007). Within the framework of the SEBI 2010 project (Streamlining European Biodiversity Indicators 2010) (Biała et al. 2012), the European Environment Agency compiled a list of the 100 most aggressive alien species for Europe (TOP-100 Europe) that threaten biodiversity (European Environment Agency 2007; DAISIE 2009; Nentwig et al. 2018). In addition, there are other national and regional lists of the most dangerous IAS. These lists have been prepared for European countries including Czech Republic, France, Italy, and Spain; and regions including the Mediterranean, Northern Europe and Baltic regions (DAISIE 2009). In particular, the international network NOBANIS (North European and Baltic Network on Invasive Alien Species, http://www.nobanis.org) includes the most dangerous IAS (TOP-82 NOBANIS) of the participant countries of this system (Austria, Belgium, Belarus, Czech Republic, Denmark, Estonia, Faroe Islands, Finland, Germany, Greenland, Iceland, Ireland, Latvia, Lithuania, Netherlands, Norway, Poland, Slovakia, Sweden, and Russia). In the NOBANIS network, Russia is represented as part of the shortened version of the database of alien species of the European part of Russia (NOBANIS 2021).

The full alien species database (DB) for the entire territory of Russia is registered in the international system Global Register of Introduced and Invasive Species (GRIIS), and published on the Global Biodiversity Information Facility (GBIF) portal (www.gbif.org) (Petrosyan et al. 2020a). The DB contains a list of 1,347 alien species from Russia. This list includes 19 taxa of alien species that have naturalized and are expanding their range (Petrosyan et al. 2017).

Despite the fact that there are general patterns in biological invasions and there are widely dispersed alien species, each country has its own particular features of invasions

and its own list of alien species, including the most dangerous (priority) species. In such a vast country as Russia, which covers an area of more than 17 million km^2 or about 1/8 of the Earth's land (Borodko 2020), invasions of many taxa occur at a large scale and require a comprehensive analysis of invasion patterns. Despite a great threat of biological invasions, there are not enough current, accurate data on the location of IAS in Russia. Although some regional or species/taxon-specific studies have been conducted (Alekseev 1989; Nedoluzhko 1997; Kuznetsov 2005; Berezina 2007; Bobrov et al. 2008; Vinogradova et al. 2009; Khlyap and Warshavsky 2010; Semenov 2010; Yakovleva and Yakovlev 2010; Kolyada 2011; Maslyakov and Izhevsky 2011; Kuzmin 2012; Antonova 2013; Gninenko et al. 2014; Ilyukh 2014; Kosoy et al. 2015; Devyatova et al. 2016; Ebel et al. 2016; Cherpakov 2017; Kukushkin et al. 2017; Karpun 2019; Khlopkova et al. 2019; Kudaktin and Romashin 2019; Petrosyan et al. 2019b; Shiganova et al. 2019; Khlyap et al. 2021a, b), a comprehensive database of occurrence records of invasive species is lacking at the national level.

A list of the 100 most dangerous IAS that pose a great threat to ecosystems and human health in Russia was published in 2018 (Dgebuadze et al. 2018). The main criteria (serious impact on biodiversity and human health at the local and regional levels) used for selection of the TOP-100 IAS among 1,347 alien species are presented in Suppl. material 1: TOP-100 selection criteria (DAISIE 2009; Dgebuadze et al. 2018). The TOP-100 Russian IAS include representatives from six kingdoms: Bacteria, Chromista, Fungi, Plantae (vascular plants), Protozoa (alveolates), Animalia (ctenophores, nematodes, molluscs, arthropods – crustaceans and insects, chordates – ascidians, ray-finned fishes, amphibians, reptiles, birds and mammals). The description of each species from the TOP-100 list is given in Dgebuadze et al. (2018), and includes species taxonomic position, the main species name and synonyms, native (historical) and invasive parts of the range, habitats, biology, its impacts on native species, ecosystems and humans, control methods, and geographical maps of its distribution. Despite the importance of the publication of the TOP-100 IAS, many aspects of the invasion process are not available in this book (Dgebuadze et al. 2018). In particular, it does not contain the diversified modern data analysis to assess the potential threat of IAS impacts on ecosystems including actual SOR, species distribution models (SDMs) and information about number of dangerous invasive species in different regions of Russia.

To fill these gaps, we created a database of SOR to identify the regions where IAS have been established in Russia. Construction of SDMs allows us to identify environmentally suitable regions for IAS, and thereby to predict potential risks of invasions (Wiens et al. 2009; Guisan et al. 2014). These models also help assess the risks of invasions of IAS and identify areas where alien species can potentially introduce, establish, spread and cause significant damage (Beukema et al. 2018).

The objectives of this research were to (1) create databases of species occurrence records for the TOP-100 IAS in Russia; (2) establish pathways, time of introduction and rates of accumulation of the TOP-100 IAS in Russia; (3) reveal the main types of spatial distributions of invasive species and their relationship with residence time; 4) distinguish regions with the highest IAS diversity and perform hot spot analysis of current distributions based on SOR.

Materials and methods

Assessment of the parameters of the species invasions

Taxonomic diversity and ecological groups of IAS

In total, we identified 1,347 alien species in Russia from which we selected the TOP-100 most dangerous IAS (see Introduction). We studied this TOP-100 IAS in Russia using a factographic database (**FDB**) of alien species. We regard the database of alien species as a factographic database because it is used for the collection and storage of important "facts" about each invasive species, including year of introduction, pathway of introduction, impact mechanism, impact output, native range, donor territory, etc (Suppl. material 2). We grouped the 100 species according to their taxonomy into 16 different taxonomic ranks: Bacteria (two species), Chromista (three species), Fungi (three species), Alveolata (one species), Vascular plants (29 species), Ctenophora (one species), Nematoda (two species), Mollusca (12 species), Crustacea (12 species), Insecta (15 species), Ascideacea (one species), Actinopterygii (five species), Amphibia (one species), Reptilia (one species), Aves (two species) and Mammalia (ten species) (Suppl. material 3).

In addition, we divided the species into five ecological groups: "microorganisms" (bacteria, chromists, fungi, alveoli, nematodes, 11 species), plants (vascular plants, 29 species), aquatic organisms (ctenophores, mollusks, crustaceans, ascidia, ray-finned fish, 31 species), terrestrial ectotherms (insects, amphibians, reptiles, 17 species) and endotherms (birds and mammals, 12 species).

Pathways, and accumulation rates of introduction, impacts of IAS on biodiversity and various sectors of the economy

The FDB of the TOP-100 species was used to assess parameters of the invasion process, including pathways of introduction, description of the native range, and to catalog which IAS have multiple impacts on hydropower, agriculture, forestry, fisheries and hunting area and human health. We also used FDB to estimate the accumulation rate of the TOP-100 IAS in Russia over time. The main trends in IAS introductions were determined based on the first record of establishment for each species in Russia. We used these data to construct regression model describing the dynamics of the number of IAS introductions over time.

Methods for constructing spatial distribution models (SDMs) using MaxEnt

The analysis consisted of four stages: (1) collection of vector database of species occurrence records and raster data of environmental variables; (2) selection of environmental variables and minimizing spatial autocorrelation (**SAC**); (3) assessment of the optimal parameters of the maximum entropy (**MaxEnt**) models according to the Akaike information criterion (**AICc**); and (4) construction of species distribution models (**SDMs**) using the MaxEnt method.

Collection of vector database of SOR and raster data of environmental variables

A vector database (VDB) was created in ArcGis Desktop 10.6.1 (ESRI 2017) using our original field data on SOR in combination with literature sources and museum collections, mainly from the Zoological Museum of MV Lomonosov Moscow State University, the museum of the Zoological Institute, Russian Academy of Sciences, DP Syreishchikov Herbarium of Moscow State University, Depository of Living Systems "Noah's Ark" of Moscow State University, and the Herbarium of the Botanical Institute of the Russian Academy of Sciences. Species occurrence records of the TOP-100 Russian IAS in North and South America, New Zealand, Western Europe, Southeast, South and Central Asia were obtained from the Global Biodiversity Database (**GBIF**) (www.gbif.org), Centre for Agriculture and Bioscience International (**CABI**) (www.cabi.org), Ocean Biodiversity Information System (**OBIS**) (obis.org), International Union for Conservation of Nature (**IUCN**) (www.iucnredlist.org), AquaMap (www.aquamaps.org), and early detection and distribution mapping system (www.eddmaps.org). SOR were collected from scientific papers and/or international sources of open access. We distinguished three types of SOR. The first type contained SOR described by accurate geographic coordinates. For this data type we first removed duplicates, and then we applied accuracy control filters (i.e. SOR that had accuracy of location of more than 5 km were excluded). Several taxa included in the TOP-100 list have subspecies rank. In this case, the SOR of the subspecies not listed in the TOP-100 were excluded. The second type included SOR whose locations are depicted in the maps without indicating accurate coordinates. For this type of data, geographic coordinates were identified using basemaps of Russia by procedures of geo-registration and binding the available species localities to basemaps using at least 30 control points chosen in the ArcGis Desktop 10.6.1 environment. Basemaps were obtained from Natural Earth of public domain map datasets (https://www.naturalearthdata.com/downloads/10m-cultural-vectors/10m-admin-0-countries). For the third type of data, we used only SOR that allowed us to determine the accurate geographic coordinates using GoogleEarth (earth.google.com) with an accuracy of at least 5 km.

We obtained a final set of SOR data by combining all three types of records after excluding duplicate records for species locations. Each SOR contained data collected over a period of twenty years (for some species, such as the *Apodemus agrarius*, over a period of 80 years) during which the species geographical distribution have been studied including species identity, accurate geographic coordinates and year of the first record. In total, there were 169,679 SOR, of which 100,613 were from the native part of the IAS range and 69,066 were from the invasive range (Suppl. material 3).

Spatial bioclimatic variables (**BIOCLIM**) with the numbers from 1 to 19 (Bio1–Bio19) were taken from a global dataset WorldClim 2.1 (http://worldclim.org/version2) with a resolution of 2.5 arc minute (~ 5000 m) (Fick and Hijmans 2017) for the historical climate. We used layers on a global scale because the native habitats of the TOP-100 IAS in Russia are located all over the world, including North and South America, New Zealand, North Africa, and Eurasia (Suppl. material 3). BIOCLIM variables characterize annual trends, seasonality, and fluctuations of temperature and

precipitation that commonly affect species distributions (Root et al. 2003; Bellard et al. 2013). In the case of freshwater species, many studies indicated strong correlations between spatial structures and climatic variables (Jocque et al. 2010), mainly temperature and water access. BIOCLIM variables were used to construct SDMs for various near-water and freshwater species (Drake and Bossenbroek 2004; McNyset 2005; Bellard et al. 2013; Banha et al. 2017).

In addition, we created raster layers of environmental variables in marine environments using the MARSPEC databases (Ocean climate layers for marine spatial ecology) (Sbrocco and Barber 2013). These layers are required for construction of SDMs of marine species. MARSPEC databases contain information related to the topographic complexity of the seabed, sea surface temperature and salinity. All raster layers of environmental variables BIOCLIM and MARSPEC datasets were prepared with resolution of 2.5 arc minute in *.asc and *.geotif formats.

Selection of environmental variables and minimizing SAC

The selection of variables for the modeling was carried out using a two-step procedure. At the first step, we created raster layers for the model-training regions for each environmental variable using the BIOCLIM or MARSPEC datasets. The model-training regions were described by the minimal convex polygons that included occurrence records reported for the native and invasive ranges (Rodda et al. 2011). The training areas were chosen based on the native and invasive regions of the species location including Russian regions (European part of Russia, Asian part of Russia – Ural, Siberia, Far East), regions of North America, Central and northern parts of South America, Western Europe, Central, Eastern, South Asia. At the second step, variables from the two sets of data, BIOCLIM and MARSPEC, were tested. We then selected variables for models using the ENMtools R-package (Warren et al. 2010). Those variables between which the pairwise Spearman correlation coefficient was greater than 0.75 in absolute value were excluded. Multicollinearity was assessed by the VIF (Variation Inflation Factor) using the Usdm R-package (Naimi et al. 2014). The advantage of the Usdm package for VIF assessment was described by Guisan et al. (2017). The environmental variable was regarded as multicollinear and excluded from the model if VIF > 5 (Hair et al. 1995; Guisan et al. 2017).

This two-step procedure led us to select six environmental variables from the BIOCLIM (Fick and Hijmans 2017) and MARSPEC (Sbrocco and Barber 2013) databases, which were used to construct SDMs for land (terrestrial and freshwater) and marine species, respectively. Environmental variables from the BIOCLIM set involved Bio1 (annual mean temperature), Bio5 (max temperature of warmest month), Bio11 (mean temperature of coldest quarter), Bio12 (annual precipitation), Bio13 (precipitation of wettest month), Bio14 (precipitation of driest month). Environmental variables selected from the MARSPEC dataset included Bathymetry (depth of the seafloor), Biogeo05 (distance to shore), Biogeo08 (mean annual sea surface salinity), Biogeo11 (annual range in sea surface salinity), Biogeo13 (mean annual sea surface temperature) and Biogeo16 (annual range in sea surface temperature).

There is no well-established methodology for accounting for SAC to build models based on only occurrence records (i.e. presence-only data) (Elith and Leathwick 2009; Guisan et al. 2017). We used an approach which involved random filtration of SOR to establish minimum spatial distances between the records (De Marco et al. 2008; Nuñez and Medley 2011; Marcer et. al. 2012; Václavík et al. 2012). Afterwards, we tested for SAC of model residuals. Many IAS have a large number of SOR. Therefore, they differ by spatial density depending on the number of samples collected in the native and invasive ranges. For this reason, we selected SOR using the procedure of random filtration of subsamples using the spThin package (Aiello-Lammens et al. 2015). This procedure is designed to (1) decrease the spatial bias of SOR located in different parts of the species range and (2) reduce the SAC of residuals of the model using approaches described by Dormann et al. (2007). We prepared two groups of datasets for each species. The first group of datasets included all available SOR without filtration. The second group consisted of 17 datasets, each of which included randomly selected SOR with different minimum distances. For the creation of these datasets we used minimum spatial distances in the range from 35 to 595 km at 35 km step. The MaxEnt models (stages three and four see below) constructed using these datasets were then tested for significance of autocorrelation of model pseudo-residuals (1 – probability of occurrence generated by model) by calculating Moran's I at multiple distance classes via the Ape (Paradis and Schliep 2019) and LetsR (Vilela and Villalobos 2015) packages. The significance was determined using permutation tests (Vilela and Villalobos 2015). We further used only those models that had a P-value greater or equal to 0.05.

Determination of the MaxEnt models' parameters

Although the MaxEnt default parameters for SDM are based on a large set of empirical data (Phillips and Dudik 2008), recent studies have shown that these models can be ineffective (Radosavljevic and Anderson 2014; Halvorsen et al. 2016). For this reason, we used three threshold-independent evaluation metrics: AUC_{Test}, AUC_{Diff}, and the Akaike size-adjusted information criterion (AICc) to select optimal MaxEnt parameters using the R-package ENMeval (Muscarella et al. 2014). AUC_{Test} is a metric that measures the discriminatory ability of a species distribution model using SOR that were not included in model construction. AUC_{Diff} is the difference between the AUC, calculated from the AUC_{Train} of the training set and AUC_{Test}. This metric (AUC_{Diff}) is a measure of the overfitting of the model. High AUC_{Diff} values characterize the degree of overfitting of the model (Warren and Seifert 2011). AICc – Akaike information criterion adjusted for small sample size reflects the degree of goodness-of-fit and complexity (Guisan et al. 2017). The model with the lowest AICc value is assumed to be the most appropriate model among the set of possible models. To estimate AUC metrics, we separated the calibration and evaluation SOR using the geographically structured partitioning scheme proposed by Radosavljevic and Anderson (2014) and implemented in the ENMeval R-package (Muscarella et al. 2014) (Suppl. material 4). This SOR partitioning scheme leads to more realistic and less biased estimates of SDM performance than the more traditional partitioning scheme using random k-fold. The

scheme of geographically separated SOR to determine the MaxEnt parameters is quite effective (Title and Bemmels 2018).

The ENMeval package created a series of MaxEnt models for each dataset using different regularization values and feature classes, compared them using the AICc criterion, and selected the most appropriate model. This package commonly selects a model that is less complex than the default model accepted by MaxEnt with an acceptable value of AUC_{Diff} metric (Halvorsen et al. 2016; Title and Bemmels 2018).

Construction of species distribution models (SDMs) using MaxEnt

The final IAS distribution models (**SDMs**) adapted to the historical climatic conditions were built using package MaxEnt 3.4.1 (Phillips et al. 2006) and Biomod v.2.0 R-package (Thuiller et al. 2021). The procedure for the selection of optimal parameters for these models was described above. SDMs were created as a result of 10 MaxEnt runs to randomly select test and training samples. Seventy percent (70%) of the SOR in the MaxEnt runs were used as training samples, and 30% of the records were used for testing models. For calibration of the model, we used from 1,000 to 10,000 randomly generated pseudo-absence (**PA**) points based on the number of SOR. We used a combination of the 'random' and 'sre' strategies for the generation of the PA points using the Biomod v.2.0 R package. Number of PA points was generated (as recommended by Barbet-Massin et al. 2012) according to the number (**N**) of SOR (if $N \leq 1000$ then 1000 points were selected, else 10,000 were selected).

We assessed the model performance using the Boyce (B_{ind}) index (Boyce et al. 2002; Hirzel et al. 2006) with the Ecospat R-package (Cola et al. 2017). Boyce index lacks several of the drawbacks associated with AUC index (Lobo et al. 2008). It requires only data on SOR and measures how much the predictive models differ from random distributions. The advantages of using this index have been shown in several studies (Hirzel et al. 2006; Petitpierre et al. 2012; Bellard et al. 2013; Petrosyan et al. 2019a, 2020b). We calculated B_{ind} for each of 10 model runs for each species, and then we averaged the run values to get the final estimates. The rank of each environmental variable in the SDM was estimated based on analysis of variable contributions and the jackknife method using MaxEnt. The highest rank was attributed to those environmental variables that had highest contribution in the model, i.e. had high values (> 5%) of permutation importance (**PI**) and/or high percent contribution (**PC**) (Phillips et al. 2006).

At this stage, we selected a model with $B_{ind} > 0.7$ and with insignificant SAC of residuals to compare it with the other models (see Selection of environmental variables and minimizing SAC section). If the model was accepted in terms of residuals, it was projected to the whole territory of Russia.

Assessment of actual and potential IAS invasive ranges in Russia

The first metric was evaluated using SDM, indicating environmentally suitable habitats for the introduction of the species in Russia, i.e. the potential invasive range. The

second metric obtained based on SOR specifies the regions where a species has already established. We assessed the size and geographical position of the invasive range by the number of administrative divisions of Russia: regions (oblasts), territory (krajs), republics, autonomous okrugs, autonomous oblasts and 2 cities of federal significance Moscow and St. Petersburg. The regions differ because they have different histories and/or types of administrative management. The map of Russian regions and the corresponding vector polygons (shape file) were downloaded from the Open Street Map source (www.openstreetmap.org).

Potential invasive range estimated by SDMs

First, we binarized the original SDM maps for the analysis. We transformed the probabilistic maps obtained with help of MaxEnt into binary suitable/non-suitable maps using the threshold maximizing the True Skill Statistics (**TSS**) (Guisan et al. 2017). Afterwards, we used the vector layer of geographic coordinates of the administrative regions of Russia to find the number of environmentally suitable regions for potential species invasion, using the "Extract Value" function in ArcGis Desktop 10.6.1.

Actual invasive range obtained based on SOR

To obtain the actual range of invasion, we converted vector polygons of the Russian regions (including coastal areas of the seas) into raster giving unique numbers to each polygon. From this, we then determined the regions which contained at least one occurrence record. Raster binary maps of the species occurrences were created for each species in the regions of Russia. Further, using the vector layer of geographic coordinates of the administrative regions of Russia, we found the number of regions where each species has been already naturalized using the "Extract Value" function in ArcGis Desktop 10.6.1.

Hereafter we used three categories to interpret the type of the distribution of species based on the SOR – local, regional, and wide. Species with local distributions occurred relatively close to the sites of introduction and were established in less than 33% of the regions of Russia. Species occupying more than 33% but less than 66% of the regions of Russia were considered as regionally distributed. Species with wide distributions had almost continuous distributions colonizing more than 66% of the Russian regions from the western to eastern borders of Russia.

Assessment of IAS richness in different Russian regions

IAS richness

To find the actual number of IAS, we summed the created raster binary maps showing occurrence sites of species. Afterwards, we obtained the final raster map of the species richness in different Russian regions using the R-package raster. We then created a vec-

tor map of species richness in Russian regions for hot/cold spots analysis using the final raster map of IAS richness and vector layer of geographic coordinates of the centers of Russian administrative regions.

Hot spots analysis and zoning according to assessments of IAS impact on ecosystems

To identify zones with high impacts of the most dangerous IAS on Russian ecosystems, we used an analysis of hot and cold spots and a procedure of constructing kernel density in the ArcGis Desktop 10.6.1 environment. Hot, cold and neutral spots were identified based on the Getis-Ord Gi * statistic (Andy 2005) using Z-scores and P-values. As a result, we identified three types of zones distinguished by IAS richness that we regard as a proxy of IAS impacts. The first type of zone aggregates hot spots with the highest richness of IAS, which, consequently, have a great impact on ecosystems. The second zone type aggregates cold spots indicating regions with few invasive species that have reduced impact. Neutral zone type does not belong to the above two types and are characterized by intermediate richness and intermediate impact on ecosystems. Finally, we constructed the original map of hot/cold spots with Russian territory zoning according to assessments of IAS impact on environmentally suitable ecosystems.

We constructed SDMs using R (R-version 3.6.2 2019) and R-packages Ape (Paradis and Schliep 2019), Biomod2 (Thuiller et al. 2021), Dismo (Hijmans et al. 2017), Raster (Hijmans et al. 2020), Ecospat (Cola et al. 2017), ENMeval (Muscarella et al. 2014), ENMtools (Warren et al. 2010), SpThin (Aiello-Lammens et al. 2015), LetsR (Vilela and Villalobos 2015) and Usdm (Naimi et al. 2014). In addition, we applied R-scripts presented in Hirzel et al. (2006) to assess the suitability of models using RSTUDIO v. 1.4.1106 software (RStudio 2020). The analysis of hot/cold spots, construction of the kernel density of hot/cold spots and visualization of the SDMs were conducted in the ArcGis Desktop 10.6.1 environment (ESRI 2017).

Results

General description of the database of SOR

The TOP-100 IAS of Russia were represented by 16 taxonomic groups (Suppl. material 3), of which 78 species were found in five groups: vascular plants – 29, insects – 15, molluscs – 12, crustaceans – 12 and mammals – 10 species. The other 11 taxonomic groups include only 1–5 species each (Fig. 1). We identified 169,709 SOR for the TOP-100 IAS in their native and invasive ranges, which included 1,117 for bacteria, 2,448 for chromists, 2,350 for fungi, 87,497 for vascular plants, 1,371 for alveolates, 760 for ctenophores, 407 for nematodes, 12,855 for molluscs, 11,995 for crustaceans, 3,780 for insects, 419 for ascidians, 14,746 for ray-finned fish, 5,197 for amphibians, 1,762 for reptiles, 2,279 for birds and 20,726 for mammals (Fig. 1). The number of SOR for individual species in their invasive and native ranges is presented in Suppl. material 3.

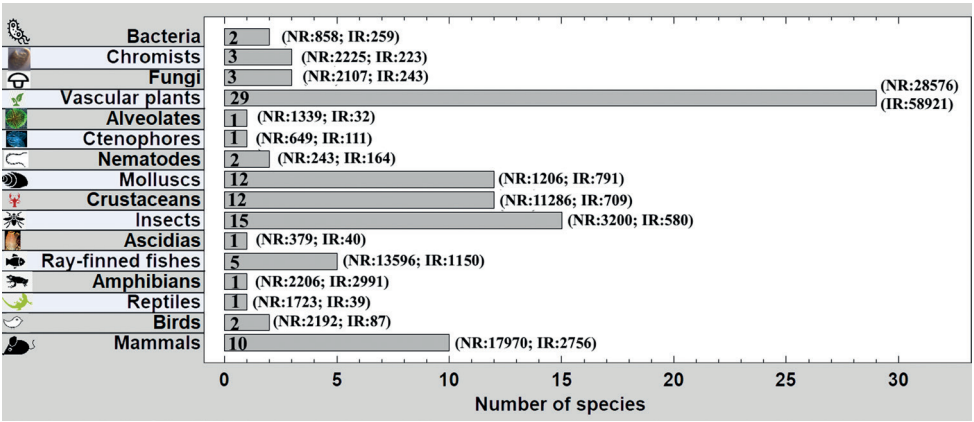

Figure 1. Species occurrence records (SOR) in the native and invasive ranges of the TOP-100 IAS in Russia, which were used to assess actual and potential IAS invasive ranges. The number of species in each of 16 taxonomic groups is indicated in the grey bars; NR and IR are the numbers of SOR in the native and invasive ranges, respectively.

The number of SOR varies widely between ecological groups. The highest number of SOR (58,921) was recorded in plants, which consists of 29 species. Most of the plant species have colonized natural, disturbed, and/or urbanized terrestrial and aquatic ecosystems in Russia (Vinogradova et al. 2009, 2018; Vinogradova and Kupriyanov 2016). American maple (*Acer negundo*) has the highest number of SOR (8,849). All occurrence records for this species of maple are located in the area from the western (including Kaliningrad Region) to the eastern borders of Russia.

A total of 3,610 SOR were reported from 17 species of terrestrial ectotherms, 3,231 SOR from 31 species of aquatic organisms, and 2,413 SOR from 11 species of terrestrial endotherms. Within these ecological groups, the highest number of occurrence records (2,691) was reported for the marsh frog (*Pelophylax ridibundus*), which are located in the southern Ural, Siberia and Kamchatka (Kuzmin 2012). The lowest number of occurrence records (921) was found for 11 species of "microorganisms". Such a low number of SOR for "microorganisms" was mainly attributed to the hard detection and species identification relative to representatives of the other groups. The highest number of records (208) in this group was reported for wetwood (*Pectobacterium carotovorum*) which were registered in all the forest areas on Russian territory except for the cold regions of Siberia (Cherpakov 2017).

Geographical IAS origin and pathways of their introduction

The greatest number of invasive species originated from America (45 species), which included North America (31 species), Central America including Mexico (3 species) and South America (2 species), the western coast of these continents with adjacent waters of the Atlantic Ocean (8 species) and the eastern coast and the Pacific Ocean

(1 species) (Suppl. material 3). One species (*Potamopyrgus antipodarum*) originated from New Zealand. *Cylindrospermopsis raciborskii* originated from either African or Australian. We were not able to establish the origins of 10 species. The native ranges of the rest of the TOP-100 IAS (43 species) were Eurasia and its surrounding waters. IAS from East Asia contributed the second most IAS to Russia (18 species). Native ranges of three species were located in Southeast Asia; two species originated from the north of the Pacific Ocean and adjacent land. Two terrestrial and one marine species invaded Russia from the south of Eurasia, two species invaded from Europe, two species came from Central Asia, and one species from the Caucasus. The Ponto-Caspian Basin was an important source of aquatic IAS (6 species) (Suppl. material 3). Among the aforementioned groups of Eurasian species, the range of 31 species at least partly includes the territory of Russia. Species living in East Eurasia, including the Russian Far East, as a rule, did not expand their range westwards but they invaded into Russian regions through Western Europe.

We found that 62% of the TOP-100 IAS were introduced into Russia unintentionally (accidentally), a third (33%) were intentionally introduced, and 5% invaded mainly by self-dispersal. The list of the TOP-100 is mainly represented by IAS carried to Russia with ballast water (22 species), cultivated plants (16 species), fouling of ships (11 species), and traffic flows (10 species).

Dynamics of accumulation of invasive species over time

The dynamics of the number of introduced IAS over time showed that there was a nonlinear relationship between the number of invasive species and year of introduction from 1600 to 2018 ($F = 2138$, $P << 0.01$, $R^2 = 98\%$) (Fig. 2, Suppl. material 3). The number of IAS that invaded Russia started to grow sharply in the middle of the 20th century. As such, the introduction of 52% of the species from the TOP-100 list occurred over a 76-year period from 1946 to 2018. Moreover, we found a significant difference ($Z = 9.5$, $P << 0.01$) in the rates at which new species were introduced (Rsy) between the two periods, namely, 1600–1945 (Rsy = 0.14 species/year) and 1946–2018 (Rsy = 0.65 species/year) (Fig. 2).

General characteristics of the impact of IAS on biodiversity and various sectors of the economy

We compiled a list of invasive species that have the greatest impact on biodiversity, various sectors of the economy, and human health (Fig. 3). Among the TOP-100 species, 36 species displace native species and 37 significantly alter the ecosystem (Fig. 3A). Seventeen species compete with native species and/or are able to displace them and five species are involved in hybridization processes. Thirty-seven species have great impacts on human health and eight species have considerable effects on hydropower systems. Some species are harmful for agriculture (29 species), forestry (20 species), fishery (13 species) and hunting grounds (8 species) (Fig. 3B).

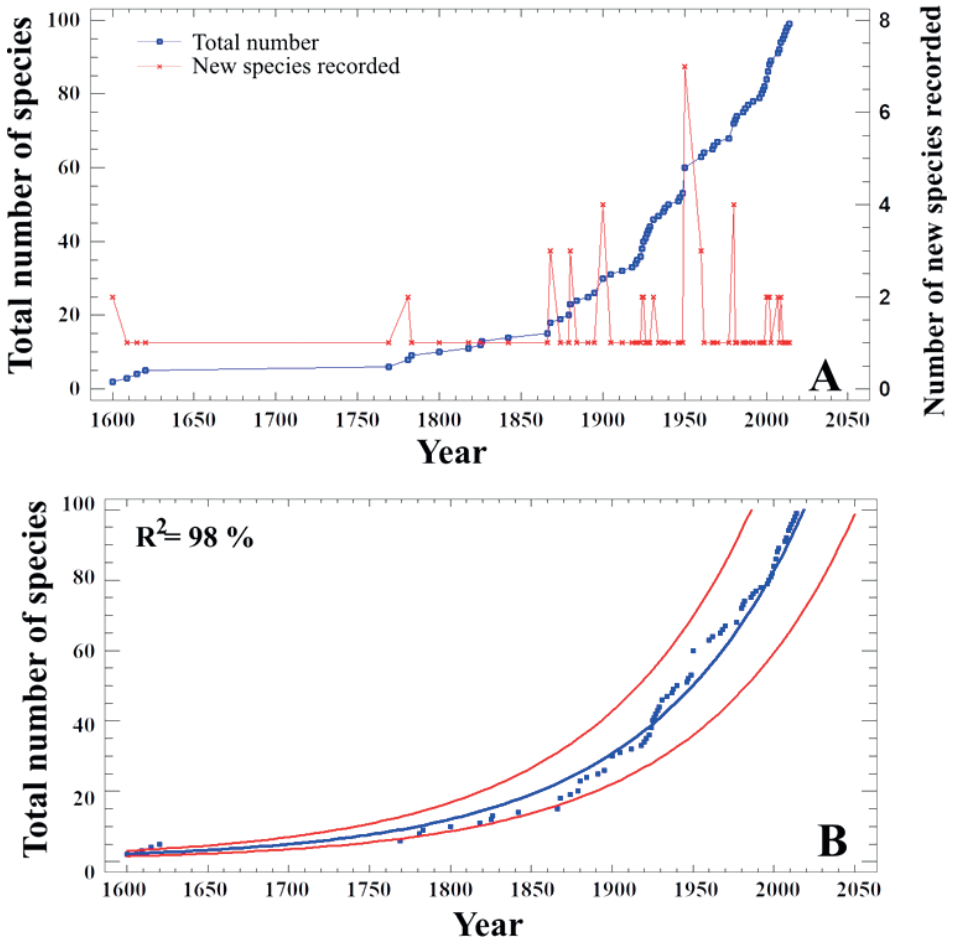

Figure 2. Number of IAS introduced during 1600–2018 in Russia (panel **A** – red) and accumulated number of species (AS) (panel **A, B** – blue dots). In the panel **B** blue curve shows nonlinear trend AS = exp (-5.73 + 0.000002536* Years^2); red lines depict 95% confidence intervals.

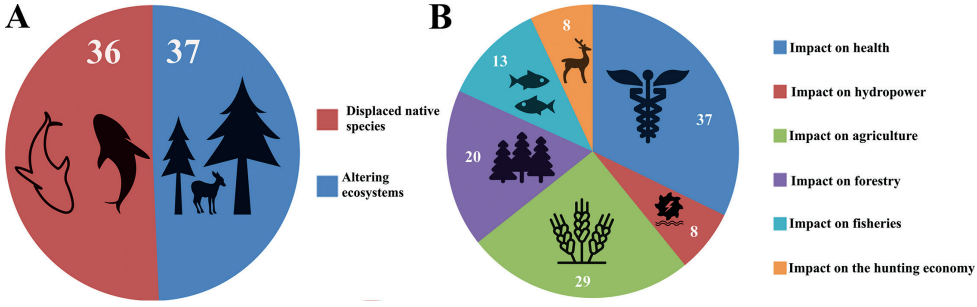

Figure 3. Impacts of the TOP-100 IAS on biodiversity, economic sectors, and human health in Russia. Panel **A** shows number of IAS altering ecosystems and displaced native species **B** shows impacts of IAS on economic sectors in Russia.

Species distribution models (SDMs)

After random selection of occurrence points, the minimum distances between the SOR points ranged from 133 to 555 km, depending on the degree of SAC for each species. This procedure reduced the number of SOR by 88%, eliminated spatial sample bias and SAC of residuals (Suppl. material 5). Furthermore, it created similar distributions of SOR in the native and invasive ranges. Moran's I correlograms for different distance classes did not show any SAC in the residuals of any of the models (Suppl. material 6). The reduced SOR data and six environmental variables from each dataset were used to construct SDMs. These models demonstrated high performance in terms of the B_{ind} index which varied from 0.76 to 0.99 with the mean 0.94 (Suppl. material 7). Based on SOR and SDMs, the studied species were divided into three groups according to their type of distribution, specifically, species with local, regional and wide distributions (Suppl. material 7). Suppl. material 7 includes short descriptions of IAS current distributions in Russia based on SOR, in particular, the number of regions in which species have already established or can potentially invade, the types of actual and predicted species distributions and model performance metrics (Boyce index – B_{ind}). Most of the IAS (56 species) currently have local distributions, slightly less (26 species) are distributed regionally, and even fewer (17 species) have wide distributions. MaxEnt models predicted a local distribution for 48 species, a regional distribution for 31 species, and a wide distribution for 20 species. Thus, SDMs predicted a decrease in the number of species (by 8) with a current local regional distribution and an increase in the number of species (by 5) with regional and wide distributions (3), respectively. The species with the widest distribution from each of 16 taxonomic groups (Suppl. material 7) are represented in Fig. 4.

The maps in Fig. 4 show that among 16 species, MaxEnt predicted local distributions for four species (Fig. 4E, F, K, N), regional distributions for seven species (Fig. 4B, C, H, I, J, M, O), and wide distributions for five species (Fig. 4A, D, G, L, P). Suppl. material 7 based on SOR data shows that among 11 less numerous species taxa, 16 IAS currently have local distributions, two species have regional distributions, and four species have widespread distributions. However, species distribution patterns are different in taxa that have a large number of IAS. In particular, a large proportion of invertebrate species have local distributions (90%), while a large proportion of vertebrate species have wide distributions (56%). For plants, the majority (66%) of species have regional distributions. A total of 78 (± 16)% of the potentially suitable regions for invasion in Russia have already been occupied (Poc) by IAS as predicted by SDMs. For species with local, regional and widespread distributions, the Poc values showed that 69 (± 15)%, 87 (± 6)% and 93 (± 4)% of potentially suitable regions had been naturalized, respectively. Thus, the largest portion of potential expansion area into environmentally suitable regions is predicted for species with local distributions (31%) and the smallest percentage of potentially expansion area is expected for species with widespread distributions (7%). For species with regional distributions, the portion of species expansion area into suitable regions is intermediate (13%).

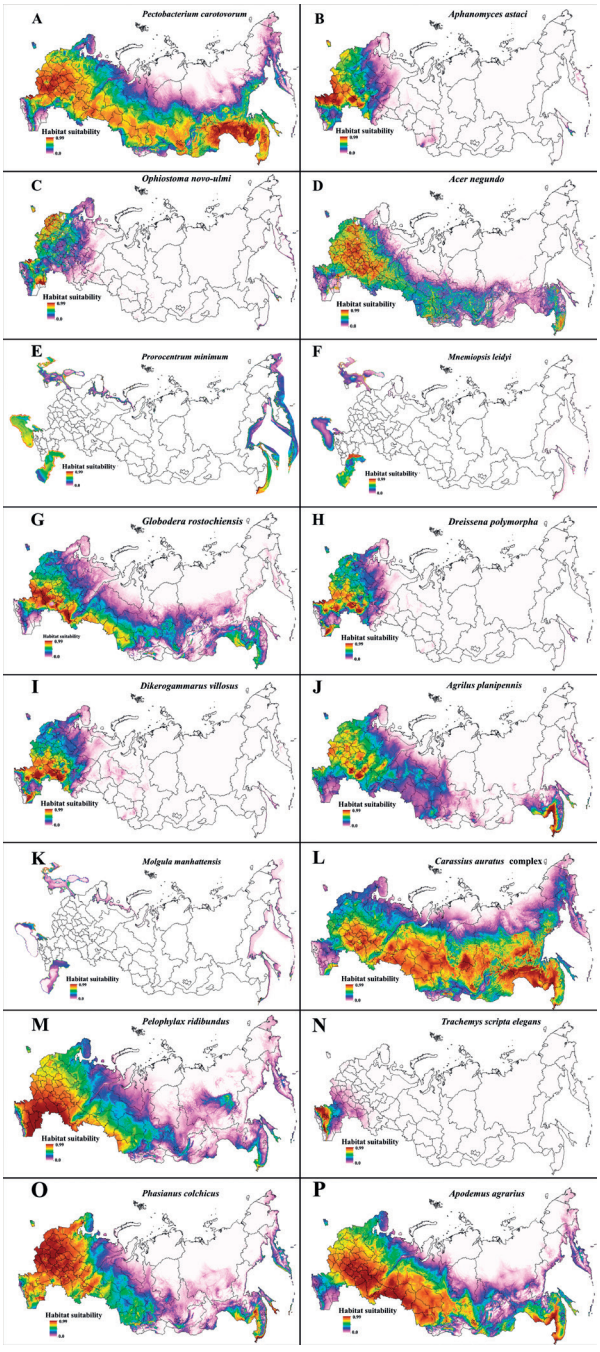

Figure 4. Predicted potential distributions of IAS including one species from the 16 taxonomic groups using SDMs. MaxEnt models used for prediction were optimized in terms of feature class and regularization according to the AICc metric. These models were constructed using selected environmental variables from the BIOCLIM or MARSPEC datasets, where species in the panels **A, D, G, L, P** have wide distribution, species in the panels **B, C, H, I, J, M, O** have regional distribution and species in the panels **E, F, K, N** have local distribution. Marine species and sea areas are shown in **E, F, K**.

Species occurrence records identified a positive relationship between the time since introduction and the type of distribution (either local, regional, or widespread), which was assessed by Spearman's rank coefficient (Src = 0.58, P << 0.01). Species with local (56 species), regional (26 species), and widespread distributions (17 species) were introduced on average 55 (± 5), 126 (± 8), and 190 (± 12) years ago, respectively. This average residence time for species with a widespread distribution is likely an underestimate because there are IAS that were introduced before the 16th century (Suppl. material 3).

SDMs showed that the environmentally suitable habitats for IAS with local distributions are mainly located in the European part of Russia. Species with regional distributions are found in the European part of Russia (9 species) and simultaneously in the European and Asian parts of Russia (22 species). The environmentally suitable habitats for the IAS with widespread distribution are located in the European part, as well as in the Asian part of Russia (Suppl. material 7).

IAS richness in different regions of Northern Eurasia

We determined the IAS richness in each administrative division of Russian territory and adjacent seas, by overlaying the SOR on the polygonal raster map (Fig. 5, Suppl. material 8). The map shows that hot spots and zones with high concentration of IAS are located in the European part of Russia (Fig. 5). In the Asian part of Russia, only cold and neutral spots were found (Fig. 5). The maximum number of species was identified in hot spots A1 (64 species, Krasnodar territory), A2 (60 species, eastern coast of the Black Sea), A3 (55 species, Leningrad region), A4 (52 species, Rostov Region) and A5 (51 species, Moscow).

In the European part of Russia, we distinguished five zones with high concentrations of hot spots (Z1 – Z5). Zones Z1 and Z2 are located in the central part of European Russia and include 23 and 7 hot spots, respectively. Zone Z3 lies in Central Ciscaucasia and includes 7 hot spots. Two smaller zones (3 hot spots in each zone) are located northwest of the European part of Russia including the adjacent part of the Baltic Sea (Z4) and in the Northwestern Caucasus including the adjacent part of the Black Sea (Z5). Cold spots are located in the north-east of the European Russia and Asian Russia (Fig. 5, Suppl. material 8). Statistical significance levels of hot, neutral and cold spots of IAS included in the TOP-100 list are confirmed by GiZScore statistics.

Discussion

We combined data in one comprehensive national database on the most dangerous TOP-100 invasive alien species in Russia, which includes data on trends and pathways of invasions, and was used to identify regions of current and predicted IAS distributions. This database included SOR in accordance to taxonomic and location quality criteria, and data on the rate of IAS accumulation over time. Although our analysis involved only 100 invasive species (7.4%) out of 1,347 IAS reported in Russia (Petrosyan et al. 2020a), these species pose the greatest threat to biodiversity and human health.

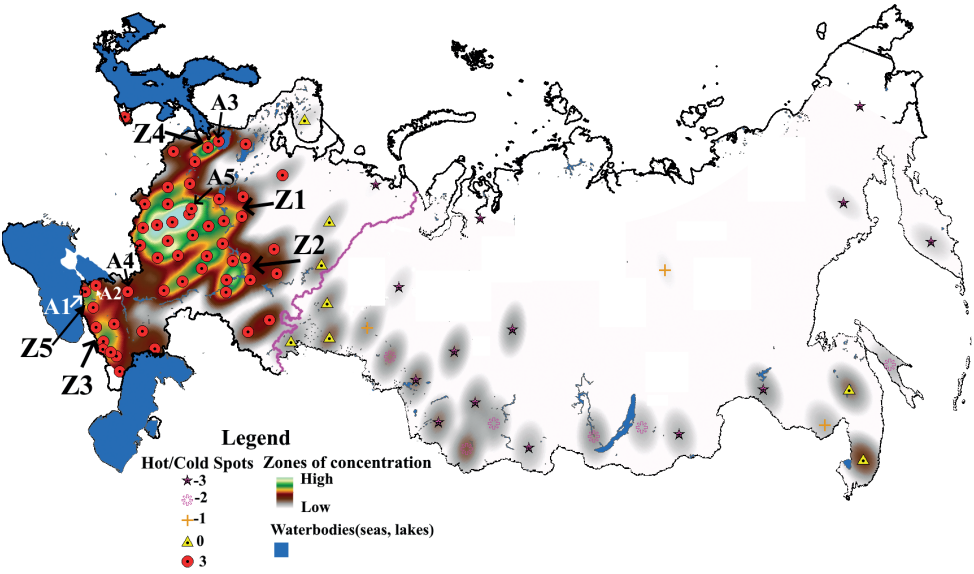

Figure 5. Hot/cold spots and zones of IAS impact on ecosystems. Cold spots with -3, -2, -1 bins reflect statistical significance with confidence levels of 99, 95 and 90% respectively, spots with 0 bins have no statistical significance. Hot spots with 3 bins reflect statistical significance with confidence levels of 99%. Symbol A denotes hot spots, symbol Z denotes zones with a high concentration of hot spots based on kernel density analysis procedure. The pink line indicates the border between the European and Asian parts of Russia.

Therefore, these species require special attention of scientists and decision-makers, which is consistent with the main objectives for the conservation of biological diversity Act before (Overview of outcomes 2011; Quick guide 2021) and after 2020 (Synthesis report 2019; Post-2020 global biodiversity framework 2020). Selection criteria for the TOP-100 in Russia (Suppl. material 1) are in full agreement with those used to identify the 100 most harmful species out of 11,000 alien species in Europe (DAISIE 2009). The TOP-100 list for Russia does not include quarantine invasive species (Kirichenko et al. 2021) because, according to Russian legislation, a quarantine species is a species that has not yet been introduced, but there is a risk of its introduction, or a species that has already been introduced but still has a limited distribution (On Plant Quarantine 2014). Species from the 1,347 species pool that were not included in the TOP-100 list either have limited distribution or their impacts on biodiversity and human health are not known. However, the invasion process is dynamic and priority IAS lists should be modified over time.

Occurrence records, invasion trends and pathways of the most dangerous IAS in Russia

The invasion process in Russia has a long history. Nevertheless, a non-linear upward trend in the dynamics of the total number of invaded species has been observed only since the late 1940s showing three peaks during 1946–1962, 1970–1981 and 2000–2014. Since

1946, the number of recorded IAS has doubled, and further increases are expected. A similar rapid growth in the number of invasive species has been documented in other countries since the second half of the 20th century, including Croatia (Nikolić et al. 2013), East Africa (Witt et al. 2018) and Romania (Sirbu et al. 2022). The above studies show that peaks in the numbers of newly introduced species occurred in Romania (Sirbu et al. 2022) and Croatia (Nikolić et al. 2013) in the 1950s, while in East Africa (Witt et al. 2018) and Russia, the maximum numbers of introduced species declined during 1940s–1960s. Common factors that facilitated the increase in introductions in the second half of the 20th century in Russia included increases in traffic flows (Kalabekov 2020) and interstate communications. Among the 14 species (represented in the TOP-100 list) that invaded Russia from 1946–1962, seven species were introduced with ballast water, fouling of ship hulls or accidentally with other aquatic organisms; four species were accidentally introduced via land routes (transport and shoots of plant culture); two species were intentionally introduced, and one species – *Castor canadensis* – invaded Russia independently from the territory of Finland.

Most of the TOP-100 IAS in Russia originated from North and Central America (45%), and the Asia-Pacific region (32%). A total of 62% of the IAS were unintentionally introduced, 33% were intentionally introduced and 5% were self-dispersed. The ratio of the number of unintentionally: intentionally introduced IAS is 1.82, which is higher than this ratio for invasive plant species in Europe (0.59) (Lambdon et al. 2008). Such a low ratio in Europe is related to the fact that the main pathways of introduction of invasive alien plant species (52% of the total number of invasive plant species in Europe) are dispersing through gardening and the use of alien plants for ornamental purposes. The horticultural industry, in particular, ornamental horticulture, is regarded as an important pathway for introduction and dispersal of alien species (Drew et al. 2010). In the TOP-100 list of IAS in Russia, the number of invasive species introduced with cultivated plants accounts for 16%. The TOP-100 list also includes a large portion of species introduced with ballast water, transport flows, ship fouling and agricultural products (58 species) (Petrosyan et al. 2017; Dgebuadze et al. 2018).

Current distribution of IAS

Local distribution

We showed that recently invaded IAS commonly have local distributions. Specifically, local distributions and a relatively short resident time of introduction are typical of 56 of the TOP-100 species (Suppl. material 7). This includes eight species of "microorganisms", three plant species, 28 aquatic species, 14 species of terrestrial ectotherms and three species of terrestrial endotherms. The mean residence time for these species is 55 (± 5) years (Suppl. material 3). MaxEnt models with high predictive accuracy according to the B_{ind} index (0.95 ± 0.04) indicated that there are 1–11 environmentally suitable regions for the potential invasion of most of the locally distributed species (Suppl. material 7). Only two aquatic species, namely, naval

shipworm (*Teredo navalis*) and red king crab (*Paralithodes camtschaticus*), were not predicted to expand further. These two species currently occupy all available coastal areas of the Black and Japan Seas (*T. navalis*) and the Barents Sea (*P. camtschaticus*) and their further dispersal may occur beyond Russian territorial waters.

Regional distribution

Among the TOP-100 IAS with regional distributions (26 species), the number of plant species (19 species) is the highest (Suppl. material 7). "Microorganisms" and aquatic organisms are represented only by one species each, and terrestrial ectotherms and endotherms include only two and three species, respectively. The mean residence time for these species is 126 (± 8) years (Suppl. material 3). MaxEnt models (B_{ind} = 0.92 ± 0.06) predict that environmentally suitable regions for potential invasions (Suppl. material 7) of IAS with regional distributions will increase by 2–11 new regions. In particular, "microorganisms" occupy six more regions, plants 3–11 more regions, aquatic organisms seven more regions, terrestrial ectotherms 5–11 more regions and endotherms 2–7 more regions.

Widespread distribution

The number of the TOP-100 species with widespread distributions is relatively small (17 species). Among them, plants had the highest number of species with widespread distributions (7 species) (Suppl. material 7). These species have similar geographical distributions and are found in all major regions of Russia, namely, the European part of Russia, the Urals, the southern parts of Siberia and the Far East. Widespread distribution was determined for two "microorganism" species and two aquatic species, one terrestrial ectotherm, and five terrestrial endotherms. The mean residence time for these species is 190 (± 17) years (Suppl. material 3). MaxEnt models (B_{ind} = 0.93 ± 0.06) predict that the number of occupied regions by widespread species will increase by 2–11, specifically, "microorganism" species will expand over 6 more regions, plants occupy 3–11 more regions, aquatic organisms 4–6 more regions, terrestrial ectotherms four more regions and endotherms 2–5 more regions.

SOR of 17 widespread species from the five taxonomic groups showed that this type of distribution is largely attributed to species that are ecologically tolerant to a large range of abiotic and biotic factors. There are also other reasons for successful distribution, including the absence of competitors, long residence time and a great variety of invasion pathways. In particular, the main pathways of plant introductions are associated with their use as ornamental plants in urban landscaping and forest belts, distribution with forage grasses, spread with ground and water transport, use in aquaculture and fishery and with the import of grain and animal food. In addition, many plant species are also dispersed by birds, small mammals and bears (Vinogradova et al. 2009, 2018; Vinogradova and Kupriyanov 2016). In general, the spread of plant invaders via anthropo- and zoochory has ensured a high level of propagule pressure that facilitates the wide geographical distribution of IAS plants (Vinogradova et al. 2009, 2018).

The vast range of the bacterium *P. carotovorum*, nematode *G. rostochiensis* and insect *L. decemlineata*, are attributed to the widespread distribution of their hosts and the high rates of development of forestry and agriculture (Butorina et al. 2012; Cherpakov 2017; Dgebuadze et al. 2018).

Dispersal of fish (*C. auratus*, *P. glenii*) and mammals (*N. vison*, *O. zibethicus*) have different patterns. They (fish and mammals) were introduced, often repeatedly, into many primary areas (Bobrov et al. 2008; Khlyap and Warshavsky 2010; Reshetnikov 2010; Vekhov et al. 2018). For example, two species of mammals (*N. vison*, *O. zibethicus*) were deliberately introduced as a fur resource. Approximately 19,000 American minks and more than 330,000 muskrats were released from 1928 to 1970 in different regions of Russia (Bobrov et al. 2008). Subsequently, these foci merged into a single vast range as a result of further self-dispersal.

Zoning of Northern Eurasia according to assessments of IAS richness

The identification of regions with the greatest IAS impacts on ecosystems is important for controlling IAS and forecasting potential regions of ecological disaster. We focused on administrative divisions of the Russian territory because the most important functions of nature management and environmental protection are implemented mainly by regional or republican services. Dangerous IAS occur in all regions of the Russian Federation. The greatest taxonomic diversity of IAS was found in the central part of European Russia, where the highest concentration of hot spots is observed (Z1). That is related to better civil development of the region and, consequently, greater anthropogenic transformation of this territory (Zubarevich 2010). The second most important zone of high IAS concentration is the Middle Volga region (Z2). After the construction of hydraulic structures linking the basins of the Baltic, Black and Caspian Seas into a single system, corridors of aquatic species spreading between the north (Baltic Sea) and the south converge in the middle reaches of the Volga (Slynko et al. 2002) to increase the number of IAS in this region. In our opinion, this invasion corridor contributed to form two hot spots in the Baltic (Z4) and Black Seas (Z5) regions. The increase in the concentration of hot spots in the south of European Russia (Z3 and Z5) is associated additionally with more favorable natural conditions for IAS, mainly with warmer climate. The identification of hot spots zones based on SOR would allow decision-makers to purposefully minimize threats from IAS, constrain their spread and/or eradicate IAS from the regional pool.

MaxEnt model predictions of IAS potential distribution

We followed best practices in model development (see in Fitzpatrick et al. 2006; Broennimann et al. 2007; Dormann et al. 2007; Kühn 2007; Beaumont et al. 2009; Guisan et al. 2014; Muscarella et al. 2014; Radosavljevic and Anderson 2014; Guisan et al. 2017; Petitpierre et al. 2017; Liu et al. 2020; Pili et al. 2020) to create SDMs for terrestrial, freshwater, and marine IAS. As a result, we were able to predict IAS

distribution with high accuracy and without SAC in the residuals. Therefore, we strongly suggest using SDM and associated maps to predict expansion of IAS ranges into suitable regions.

Further species dispersal to environmentally suitable regions in accordance with predictions of MaxEnt models, is highly probable because measures to prevent self-dispersal and/or to restrict abundance were applied only for 19 species from the TOP-100 IAS in Russia (Dgebuadze et al. 2018). Control methods have been developed for 62 species including mechanical, chemical or biological approaches. Yet, there are no control methods to constrain population growth for 38 species either in Russia or in the world (Clout and Williams 2009; DAISIE 2009; Isaev 2015; Dgebuadze et al. 2018).

Conclusions

Although there is a long history of species invasions in Russia, the number of introduced IAS has been growing non-linearly over the past 76 years. The TOP-100 list is represented by 62% species that were unintentionally introduced (imported) with ballast water, traffic flows, ship fouling, agricultural products, cultivated plants and plants for landscape design. Intentional introductions have contributed much less to the invasion of IAS in Russia (33%). The majority of IAS recorded in Russia originated from North and Central America (45%) and the Asia-Pacific region (32%).

The database of actual SOR in individual regions of Russia and SDM maps allowed us to distinguish three types of distributions of the TOP-100 IAS which included local (56 species), regional (26 species) and widespread (18 species) distributions. We found that species that are widely distributed in Russia were introduced more than 190 years ago, species that are regionally distributed appeared in Russia 126 years ago, and species that are locally distributed first arrived 55 years ago.

We identified zones with high concentrations of IAS where the potential impact of IAS on terrestrial and aquatic ecosystems was the highest. These zones are located mainly in the more developed parts of European Russia with strong trade links and in the southern warm regions including the coasts of the Black Sea. We propose regularly updating SOR databases that can serve as a valuable tool in the management of biological invasions at the national and regional levels. It is noteworthy that the database of SOR at the geopolitical/regional subjects' level and MaxEnt models can be used for estimating rates and dynamics of IAS dispersal.

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Supplementary material 1

Criterion selection of the TOP-100 IAS

Authors: Varos Petrosyan, Fedor Osipov, Irina Feniova, Natalia Dergunova, Andrey Warshavsky, Lyudmila Khlyap, Andrew Dzialowski

Data type: text (Pdf file)

Explanation note: The main criterion for selecting the TOP-100 invasive alien species (IAS).

Link: https://doi.org/10.3897/neobiota.82.96282.suppl1

Supplementary material 2

General description and conceptual structure of the database (FDB)

Authors: Varos Petrosyan, Fedor Osipov, Irina Feniova, Natalia Dergunova, Andrey Warshavsky, Lyudmila Khlyap, Andrew Dzialowski

Data type: figure (Pdf file)

Explanation note: General description of the factographic database (FDB) of alien species in Russia and functional links between master and reference tables (fig. S1).

Link: https://doi.org/10.3897/neobiota.82.96282.suppl2

Supplementary material 3

Species native range, introduction year, occurrence records

Authors: Varos Petrosyan, Fedor Osipov, Irina Feniova, Natalia Dergunova, Andrey Warshavsky, Lyudmila Khlyap, Andrew Dzialowski

Data type: table (xlsx file)

Explanation note: Species native range, introduction year in Russia, number of occurrence records in the native and invasive ranges and years of creation of datasets (sheet 1), DOIs of used datasets (sheet 2), full datasets of species occurrence records (sheet 3).

Link: https://doi.org/10.3897/neobiota.82.96282.suppl3

Supplementary material 4

Geographic partitioning of the SOR

Authors: Varos Petrosyan, Fedor Osipov, Irina Feniova, Natalia Dergunova, Andrey Warshavsky, Lyudmila Khlyap, Andrew Dzialowski

Data type: figure (Pdf file)

Explanation note: Geographic partitioning of the SOR (using *Acer negundo* as an example).

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Supplementary material 5

Moran's I indexes of residual spatial autocorrelation for MaxEnt models

Authors: Varos Petrosyan, Fedor Osipov, Irina Feniova, Natalia Dergunova, Andrey Warshavsky, Lyudmila Khlyap, Andrew Dzialowski

Data type: table (xlsx file)

Explanation note: Moran's I indexes of residual spatial autocorrelation for MaxEnt models predicting the distribution of IAS in Russia and adjacent territories. These models were calibrated based on the SOR reported in the native and invasive range

Link: https://doi.org/10.3897/neobiota.82.96282.suppl5

Supplementary material 6

Moran's I correlograms of residual spatial autocorrelation for MaxEnt models

Authors: Varos Petrosyan, Fedor Osipov, Irina Feniova, Natalia Dergunova, Andrey Warshavsky, Lyudmila Khlyap, Andrew Dzialowski

Data type: figure (Pdf file)

Explanation note: Moran's I correlograms of residual spatial autocorrelation for MaxEnt models predicting the distribution of IAS in Russia and adjacent territories. These models were calibrated based on the SOR reported in the native and invasive range

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Supplementary material 7

The short description of invasive range of IAS in Russia

Authors: Varos Petrosyan, Fedor Osipov, Irina Feniova, Natalia Dergunova, Andrey Warshavsky, Lyudmila Khlyap, Andrew Dzialowski

Data type: table (xlsx file)

Explanation note: Short description of invasive range of IAS in Russia, current and potential types of distribution, and productive accuracy of MaxEnt SDMs (Bind±SE).

Link: https://doi.org/10.3897/neobiota.82.96282.suppl7

Supplementary material 8

Species richness of IAS in Northern Eurasia

Authors: Varos Petrosyan, Fedor Osipov, Irina Feniova, Natalia Dergunova, Andrey Warshavsky, Lyudmila Khlyap, Andrew Dzialowski

Data type: table (xlsx file)

Explanation note: Species richness of terrestrial and aquatic IAS in the Russian regions and assessment of significance of hot and cold spots using Gi statistics.

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Genetic diversity and structure of *Crupina vulgaris* (common crupina): a noxious rangeland weed of the western United States

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Abstract

Common crupina (*Crupina vulgaris*) is a federal noxious weed in the western USA that is currently the target of a classical biological control programme using the fungus *Ramularia crupinae*. We first identified and determined the location of populations of the two varieties of common crupina in the western United States and assessed the pattern of genetic diversity and structure of these populations. We found seven AFLP (Amplified Fragment Length Polymorphism) genotypes for 326 plants in 17 populations. AFLP genotypes correlated with two taxa, either *C. vulgaris* var. *vulgaris* or *C. vulgaris* var. *brachypappa*. This annual species is outcrossing, but relies on selfing when pollination does not occur, which may explain why less than 1% of the genetic variation is within populations. We found strong population genetic structuring and can typically predict genotype or variety for a given location. Researchers and managers will be able to predict and survey for differential efficacy of *R. crupinae* on the different genotypes and varieties during initial biological control field releases, thereby increasing the likelihood of successful biocontrol establishment and impact.

Keywords

AFLP, common crupina, *Crupina vulgaris*, biological control

Introduction

A plant invasion can be a very diverse collection of genotypes ranging across a large landmass and be present in multiple, diverse ecological situations. Different lineages of an invasive species, reflecting different evolutionary origins and phenotypes, can be present (Ward et al. 2008) and the resulting complex variation in plant traits can exist at large and small spatial scales. The identification of traits that facilitate the spread of invasions and the interactions amongst these traits, are fundamental challenges in invasion ecology (Pyšek et al. 2015) and important in the development of effective management strategies. Knowing the distribution of varying genotypes across an invaded range can be critical for managing the invasion, especially if phenotypes vary in how they invade or react to weed control methods (Ward et al. 2008; Williams et al. 2020). A classical biological control agent can have different rates of efficacy on different host plant genotypes. This has occurred with fungal agents used in the control of rush skeletonweed (*Chondrilla juncea*; Burdon et al. (1981)), a mite agent of old world climbing fern (*Lygodium microphyllum*; Goolsby et al. (2006)) and insect agents of Brazilian peppertree (*Schinus terebinthifolius*; Manrique et al. (2008)). Similarly, agents may have cryptic subspecies or genotypes that behave differently on the same plant genotype (see examples in Smith et al. (2018)). Mismatching agent and plant genotype can reduce biological control efficacy or lead to a failed release. If the invasive plant species has strong population structuring, i.e. the genotype can be predicted by location, releases can be planned to place the most efficacious agent on the appropriate plant genotype.

Common crupina (*Crupina vulgaris* Pers. ex Cass., Asteraceae) is a federal-listed noxious weed in the USA (USDA APHIS 2022). It is an overwintering annual plant with origins in the Mediterranean Region (CABI 2022). Common crupina is a close relative of the knapweeds (*Centaurea* spp.) and competes with grasses and forbs in grazing and natural areas (Miller and Thill 1983). There are two varieties of common crupina established in the USA: *C. v.* var. *vulgaris* (often incorrectly named *C. v.* var. *typica*) and *C. v.* var. *brachypappa* P. Beauv. (Latin for short pappus; the feather-like hairs on the seed), that can be reliably separated by rosette form and seed morphology (Couderc-LeVaillant 1993; Roché et al. 1997). Common crupina was first reported in the USA near Grangeville, Idaho (1969), with subsequent reports from Sonoma County, California (1975), Chelan County, Washington (1984), Umatilla County, Oregon (1987) and Modoc County, California in 1991 (Garnatje et al. 2002). It increased its range more than 1000-fold in 30 years and now occupies > 25,000 ha (Garnatje et al. 2002) and is established in multiple counties in California, Washington, Oregon and especially Idaho (EDDMaps 2022; SDA NRCS 2022).

Common crupina is an outcrossing species that attracts generalist insect pollinators with pollen and nectar, but when conditions are not favourable for cross pollination (e.g. cooler weather, low common crupina density, pollinators attracted to other plant species etc.), common crupina relies on selfing (i.e. self-pollination) to produce seeds (Couderc-LeVaillant 1984) without notable loss in fecundity (Roché 1996). It is unknown which mating system dominates in the invasion. The mating system has a bearing on invasion

success; sexual reproduction via outcrossing provides new genetic combinations, which may be selected for when environments are variable or when expanding range into different environments. Plants that rely mainly on selfing have reduced genetic variation, but may have an advantage for persisting in environments similar to their parental origins and a single or few individuals can reproduce and start a population without relying on pollen from another individual (Barrett et al. 2008; Razanajatovo et al. 2016). Knowledge of the mating system in an invasion allows better niche targeting (what part of the plant to attack) when selecting biological control agents (Gaskin et al. 2011).

This weed species is currently a target of classical biological control using the federally approved leaf- and stem-spotting fungus, *Ramularia crupinae* Dianese, Hasan & Sobhian (Deuteromycotina) (Bruckart et al. 2014). Previous genetic studies investigating the origins and invasion of common crupina were performed on five populations (five plants per population; Roché et al. (2003) and Garnatje et al. (2002)) and showed that the two varieties are genetically distinct. Moreover, accessions of *C. v.* var. *brachypappa* showed significant differences in susceptibility to a previously proposed, non-approved biological control agent, *Puccinia crupinae* Ranoj. (Bruckart et al. 2006). Due to the differential susceptibility of varieties and genotypes to fungal attack, the goals of this research are to support the newly-approved biological agent, *R. crupinae*, with a more extensive molecular analysis of the genetic diversity and population structure of the common crupina invasion. Our specific objectives were to: 1) determine distribution of taxonomic varieties using morphological characteristics and genetic data; and 2) describe the amount and structure of genetic diversity within and amongst the two varieties and invasive populations of common crupina in the western USA invasion.

Methods

Leaf material was collected from 326 plants (17 locations, mean = 19.2 plants per location) (Fig. 1, Table 1, Suppl. material 1; Population Data tab). Our survey of populations was relatively complete, with no other known invasions in California, Washington or Oregon. There may be other populated counties in Idaho (EDDMaps 2022), but we were unable to find or obtain specimens from Adams, Bingham, Fremont, Gem or Washington Counties. Plants were sampled haphazardly across an invasion patch and at least 1 m apart, except for population 17, which was received from the USDA ARS laboratory in Ft. Detrick, Maryland and was sourced from Chelan County, WA in 2001. We extracted genomic DNA from approximately 20 mg of silica-dried leaf material using a modified CTAB method (Hillis et al. 1996). Our amplified fragment length polymorphism (AFLP) method followed Vos et al. (1995) with modifications as in Gaskin and Kazmer (2009). All 15 selective primer combinations of MseI + CAA, CAC, CAT, CTA or CTA and EcoRI + AAG, ACC or ACT were pre-screened for PCR product quality and number of variable loci using eight plant samples and the two primer pairs with the most polymorphic loci were chosen (MseI + CAT/ EcoRI + ACT and MseI + CAC/ EcoRI + AGG). AFLP data were generated on an Applied Biosystems (ABI, Foster City,

Table 1. Location and plant information for *Crupina vulgaris* collections.

Population	State	County	Location	N[1]	Genotypes present	G[2]	G/N	PLP[3]	L[4]
1[v]	CA	Sonoma	Santa Rosa	20	G1	1	0.05	0.00	
2[b]	CA	Modoc	Kelly Springs	20	G3	1	0.05	0.00	5.9
3[v]	ID	Idaho	Slate Creek	20	G2	1	0.05	0.00	7.2
4[v]	ID	Idaho	Harpster	20	G1, G2	2	0.10	0.02	7.9
5[v]	ID	Clearwater	Orofino	19	G1	1	0.05	0.00	7.4
6[v]	ID	Nez Perce	Waha	20	G1	1	0.05	0.00	8.1
7[v]	ID	Idaho	Gil Gulch	20	G1, G6, G7	3	0.15	0.04	7.8
8[v]	OR	Wallowa	Joseph Creek	20	G2	1	0.05	0.00	7.7
9[v]	OR	Wallowa	Grouse Creek	20	G1	1	0.05	0.00	
10[v]	OR	Baker	Halfway	20	G1	1	0.05	0.00	8.1
11[v]	OR	Baker	Pine Creek	20	G1	1	0.05	0.00	
12[v]	OR	Umatilla	Tollgate	20	G1, G2	2	0.10	0.02	
13[v]	OR	Umatilla	Walla Walla River	20	G1	1	0.05	0.00	
14[b]	WA	Chelan	Lake Chelan 1	20	G4	1	0.05	0.00	
15[b]	WA	Walla Walla	Biscuit Ridge	20	G5	1	0.05	0.00	
16[b]	WA	Walla Walla	Blacksnake	20	G5	1	0.05	0.00	
17[b]*	WA	Chelan	Lake Chelan 2	7	G3	1	n/a	0.00	

[1]Number of plants sampled. [2]Number of unique AFLP genotypes. [3]Proportion of loci polymorphic at the > 5% level. [4]L mean pappus length of 15 seeds (in mm). [v]*Crupina vulgaris* var. *vulgaris*, [b]*Crupina vulgaris* var. *brachypappa*. *Seeds from this collection were collected in 2001, sent to a laboratory for storage, then grown for DNA sampling; thus, this may not represent a true population in that the seed could have come from one or many plants.

CA, USA) 3130 Genetic Analyzer and any individuals that did not produce a typical electropherogram pattern (i.e. noise > 20 relative fluorescence units (rfu) or failed to produce sufficient number of peaks) were omitted. We repeated AFLPs for all unique genotypes and to estimate AFLP error rate, we performed repeats of 56 samples (17% of all samples) starting with CTAB extracted material, scored them blindly and calculated the number and percentage of mismatches between the original and repeat AFLP datasets. NTSYS-PC ver. 2.2 software (Rohlf 2005) was used to calculate the Dice pairwise similarity coefficient between AFLP genotypes. The Dice coefficient ranges from 0.0 to 1.0, with values of 1.0 indicating that individuals are genetically identical. To visually assess similarity of genotypes, we used the UPGMA clustering method on Dice similarity coefficients as implemented in the SAHN module of NTSYS to create a dendrogram of the genotypes. To determine level of diversity in a population, we calculated G/N as number of unique genotypes found, divided by the number of plants genotyped. To determine how distinct genotypes were within a population, we manually calculated PLP (Proportion of Loci Polymorphic at the > 5% level) by counting how many of the loci varied within a population. To determine population structure and amount of differentiation amongst and within taxonomic varieties and populations, we performed distance based AMOVA (analysis of molecular variance) and resulting Φ values (analogous to F values) on the binary AFLP data, using the GenAlEx add-in for Excel (Peakall and Smouse 2012) with 95% confidence intervals generated from 999 permutations. To determine taxonomic variety, we measured average pappus length for 15 samples per

population (for nine populations listed in Table 1) as in Couderc-LeVaillant (1993). We used a Student's t-test to determine if pappus length means of the two putative taxonomic varieties were significantly different.

Results

We found 47 repeatable and reliable loci using the two AFLP primer pairs listed above. We did 56 repeats for both primer pairs (i.e. 56 × 47 = 2632 cells compared) and found 0 errors. All repeated AFLPs for unique genotypes were identical to the original. Across all plants, the PLP was 100% (i.e. this indicates that all 47 loci varied across the 326 plants we analysed).

For the 326 plants, seven AFLP genotypes were identified and designated G1–G7 (Suppl. material 1; AFLP Data tab). G/N in populations varied from 0.5 (all plants are one genotype) to 0.15 (three genotypes in a population). PLP per population ranged from 0–0.04 (Table 1). Pairwise Dice similarity of the seven genotypes ranged from 0.20–0.98. Similarity values between genotypes of the two varieties ranged from 0.20–0.33 (Table 2).

Mean pappus length from populations containing G1, G2, G6 and G7 was 7.9 mm (S.D. 0.73; n = 135 seeds measured) and was similar to measurements of *C. v.* var. *vulgaris* (mean = 7.98 mm ± 0.19 mm) performed by Couderc-LeVaillant (1993). Mean pappus length from populations containing genotypes G3 and G5 (G4 not measured) was 5.3 mm (S.D. 0.74; n = 30 seeds measured) and was similar to measurements of *C. v.* var. *brachypappa* (mean = 5.14 ± 0.1 mm) performed by Couderc-LeVaillant (1993). Our means for pappus length from the two taxonomic varieties were significantly different based on the results of a Student's t-test ($t = 17.5955$, $df = 163$, $P < 0.0001$; data not shown).

Based on the UPGMA, genotypes G1, G2, G6 and G7 clustered separately from G3, G4 and G5 (Fig. 1b). Similarity within *C. v.* var. *vulgaris* ranged from 0.96–0.98, while similarity within *C. v.* var. *brachypappa* ranged from 0.67–0.73 (Table 2, Fig. 1c (UPGMA)). In the AMOVA analysis, 91.6% (Φ_{RT}) of the genetic variation was amongst taxonomic varieties, 8.2% (Φ_{PR}) was amongst populations in those varieties and 0.14% (Φ_{PT}) was found within populations ($P = 0.001$ for all values).

Table 2. Pairwise genetic Dice similarity values amongst the seven AFLP genotypes of *Crupina vulgaris* in the western USA. Shaded cells are *Crupina* var. *brachypappa*; non-shaded cells are *Crupina* var. *vulgaris*.

	G1	G2	G3	G4	G5	G6	G7
G1	-						
G2	0.98	-					
G3	0.20	0.20	-				
G4	0.33	0.32	0.73	-			
G5	0.22	0.21	0.74	0.67	-		
G6	0.98	0.96	0.20	0.32	0.26	-	
G7	0.98	0.96	0.24	0.32	0.26	0.96	-

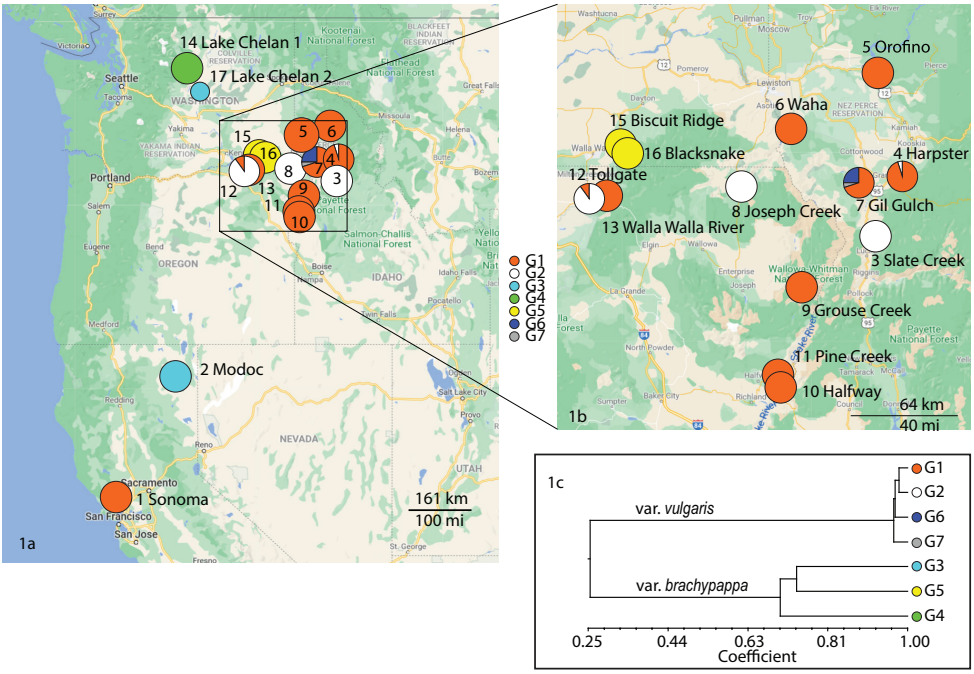

Figure 1. a Map of 17 populations of *Crupina vulgaris* and their AFLP genotypes in the western USA **b** expanded map of north-eastern Oregon, south-western Washington and western Idaho **c** UPGMA (unweighted pair group method with arithmetic mean) of the seven AFLP genotypes showing their similarity.

Discussion

Earlier studies of the origins and invasion of common crupina (Roché et al. (2003) and Garnatje et al. (2002); the same data and analysis were published in both studies) used RAPDs (Randomly Amplified Polymorphic DNA) to identify differences between genotypes. Their RAPDs identified five genotypes in five locations, while our AFLP study identified seven genotypes in 17 locations. Our G1, G2, G6 and G7 ("*vulgaris*"-type) genotypes correlate with the "Oregon", "Sonoma" California and "Lawyer Canyon" Idaho (this location was not sampled by us, but likely falls between our populations 4 and 5) genotypes of the RAPDs study, while G3, G4 and G5 ("*brachypappa*"-type) correlate with the "Modoc" California and "Chelan" Washington RAPD genotypes. The RAPDs study by Garnatje et al. (2002) found different varieties and genotypes in different USA locations and suggested this as evidence of three or more successful introductions of common crupina into the USA. Our result of seven genotypes in the USA also supports this hypothesis of multiple introductions. The earlier study of five plants per population did not note any within population variation. In contrast, three of our 17 populations had multiple (2 or 3) closely-related genotypes and the rest were monotypic populations. Both RAPDs and AFLPs are typically highly variable at the population level for plants that outcross (Powell et al. 1996). This low level of within-population variation for the invasion suggests that seed

production occurs mostly through self-pollination (selfing). Common crupina is known to self-pollinate when conditions are not favourable for outcrossing (Couderc-LeVaillant 1984), without notable loss in fecundity (Roché 1996). The low level of diversity and predominant selfing in the invasion could facilitate management success, as lower genetic diversity can suggest fewer opportunities for future evolution of resistance or tolerance to herbivory (Núñez-Farfán et al. 2007) or herbicides (e.g. Baucom and Mauricio 2004).

There are many biotic and abiotic variables regulating efficacy of classical biological control agents of weeds (Waage and Greathead 1988; McFadyen 1998). Within a species, heritable differences in resistance or tolerance to herbivory or disease can exist (Strauss and Agrawal 1999). Differential susceptibility of common crupina to a fungal pathogen has been previously observed. Bruckart et al. (2006) demonstrated that plants from Modoc, CA (our G3, var. *brachypappa*) were resistant to the fungal rust pathogen *Puccinia crupinae*, while the other accessions (Idaho, Chelan WA, Santa Rosa CA and Oregon) were susceptible. In contrast, Bruckart et al. (2014) found no significant differences in susceptibility to the leaf-spotting fungus *R. crupinae* for the two common crupina varieties when evaluated against seven crupina populations. This evidence, combined with our results, suggests baseline genetic differences amongst populations are unlikely to encumber susceptibility to the release of the biocontrol agent, *R. crupinae*.

From our seed measurement data and data from earlier studies (Couderc-LeVaillant 1993; Garnatje et al. 2002; Roché et al. 2003), we note that the genetic data correlates with previously suggested taxonomic designations. This supports a geographical structuring of regions, with *C. v.* var. *brachypappa* found in Washington and north-eastern California and all other populations being *C. v.* var. *vulgaris*. Our extensive survey of the common crupina invasion will allow researchers to test potential agent efficacy for all known genotypes prior to release. Since we found strong population structuring and can accurately predict taxonomic variety for a given location, researchers and managers can, on a local level, better predict and survey for differential efficacy during initial biological control field releases, thereby increasing the likelihood of successful biocontrol establishment and impact. Even though there was no significant difference in susceptibility to the leaf-spotting fungus *R. crupinae* between the two crupina varieties tested in laboratory conditions (Bruckart et al. 2014), efficacy of attack on the different plant genotypes in the field will be an important part of future monitoring programmes during the impending *R. crupinae* release.

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Supplementary material 1

Population Data and AFLP Data

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RESEARCH ARTICLE

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Population level interactions between an invasive woodwasp, an invasive nematode and a community of native parasitoids

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Abstract

Parasitic nematodes and hymenopteran parasitoids have been introduced and used extensively to control invasive Eurasian *Sirex noctilio* woodwasps in pine plantations in the Southern Hemisphere where no members of this community are native. *Sirex noctilio* has more recently invaded North America where *Sirex*-associated communities are native. *Sirex noctilio* and its parasitic nematode, *Deladenus siricidicola*, plus six native hymenopteran woodwasp parasitoids in New York and Pennsylvania, were sampled from 204 pines in 2011–2019. *Sirex noctilio* had become the most common woodwasp in this region and the native parasitoids associated with the native woodwasps had expanded their host ranges to use this invader. We investigated the distributions of these species among occupied trees and the interactions between *S. noctilio* and natural enemies as well as among the natural enemies. *Sirex noctilio* were strongly aggregated, with a few of the occupied trees hosting hundreds of woodwasps. Nematode parasitism was positively associated with *S. noctilio* density, and negatively associated with the density of rhyssine parasitoids. Parasitism by the parasitoid *Ibalia leucospoides* was positively associated with host (*S. noctilio*) density, while parasitism by the rhyssine parasitoids was negatively associated with density of *S. noctilio*. Thus, most *S. noctilio* come from a few attacked trees in a forest, and *S. noctilio* from those high-density trees experienced high parasitism by both the invasive nematode and the most abundant native parasitoid, *I. l. ensiger*. There is little evidence for direct competition between the nematodes and parasitoids. The negative association occurring between rhyssine parasitoids and *I. l. ensiger* suggests rhyssines may suffer from competition with *I. l. ensiger* which parasitize the host at an earlier life stage. In addition to direct competition with the native woodwasp *Sirex nigricornis* for suitable larval habitat within weakened trees, the large *S. noctilio* population increases the parasitoid and nematode populations, which may increase parasitism of *S. nigricornis*.

Keywords
Aggregation, co-infection, competition, *Deladenus*, density dependence, forest pest, *Ibalia*, parasite community, pine, *Rhyssa*, *Sirex noctilio*, spillback

Introduction

In any community there are multiple parasite species simultaneously associated with any host species (Shaw and Dobson 1995; Pedersen and Fenton 2007). Parasites share hosts by infecting different individuals in host populations and by co-infecting the same host individuals. Thus, interactions among parasites occur indirectly due to exploitative competition for shared hosts or directly through interaction outside the host (Ode et al. 2021), as well as within host individuals (van Nouhuys and Punju 2010; Harvey et al 2013). Because parasite species are each independently sensitive to host density, the strengths of their interactions with each other and with hosts can change with host density. The resulting interactions influence the population dynamics of both parasites and hosts, affecting the composition and structure of communities (Settle and Wilson 1990; Telfer et al. 2010; Holt and Bonsall 2017).

Invasive species may act as consumers, competitors, hosts to existing parasites, and as parasites of existing hosts. The introduced species may have a greater prevalence than in their native range (Callaway and Aschehoug 2000; Wolfe 2002), and their invasion can have a strong effect on the structure of native communities (Mitchell et al. 2006; Kelly et al. 2009; Kenis et al. 2009). The roles of multiple parasites, both native and introduced, in these new composite communities are understudied (Sarabeev et al. 2022) and complex (Dunn et al 2012; Llopis-Belenguer et al. 2020). For instance, in the northeastern US, the invasive spongy moth, *Lymantria dispar*, interacts with two pathogens and numerous parasitoid species that have been accidentally and intentionally introduced (Fuester et al. 2014), resulting in many complex interspecific dynamics (Hajek and van Nouhuys 2016).

Larvae of the Eurasian woodwasp *Sirex noctilio* (Siricidae) develop in the xylem of pine trees in association with a symbiotic white rot fungus, *Amylostereum areolatum*, that assists in slowly killing attacked trees and acting as an external rumen for larvae (Hajek and Castrillo 2021; Gruner and Thompson 2021). In North America, this invasive woodwasp was first found to be established near Lake Ontario in New York state in 2004 and has been spreading since then (Liebhold and Hajek 2021). *Sirex noctilio* has previously invaded and caused severe damage in pine plantations in the Southern Hemisphere, where pines are not native (Hurley et al. 2007). In North America, where pines are native, *S. noctilio* often successfully develops within trees that are already suppressed or weakened (Dodds et al. 2010; Foelker and Haavik 2021). Native *Sirex* species in North America and their associated parasite communities were already present when *S. noctilio* arrived. In particular, in eastern North America, the native *Sirex nigricornis* and associated parasitoids and nematodes already infested weakened pine trees (Table 1). Since its introduction, *S. noctilio* has been found to develop within the

Table 1. Members of the North American *Sirex* community associated with *Pinus* in this study, made up of invasive and native species and their origins. Invasives are listed in bold.

Species	Origin
Woodwasps (Siricidae)	
Sirex noctilio	**Eurasia**
Sirex nigricornis	Eastern North America
Parasitoids (Hymenoptera)	
Ibaliidae	
Ibalia leucospoides ensiger	Eastern North America
Ichneumonidae	
Rhyssa lineolata	Eastern North America
Rhyssa persuasoria	Eastern North America
Rhyssa crevieri	Eastern North America
Megarhyssa nortoni	Eastern North America
Pseudorhyssa nigricornis	Eastern North America
Parasite (Nematoda)	
Neotylenchidae	
***Deladenus siricidicola* INA**	**Eurasia** (not North America)
Sirex symbiont/*Deladenus* food (Fungus)	
Russulales	
Amylostereum areolatum	North America + **Eurasia**

same pines as *S. nigricornis* (e.g., Hajek et al. 2013, 2017) and is attacked by the same species of hymenopteran parasitoids (Foelker et al. 2016a, b) and parasitic nematodes (Morris et al. 2013; Haavik et al. 2016) (Table 1).

The community of parasitoids associated with *S. noctilio* and *S. nigricornis* in eastern North America is composed of up to six native hymenopteran species. *Ibalia leucospoides ensiger* (Ibaliidae) parasitizes eggs/early instars and is almost always the most common species. Four species of rhyssines (Ichneumonidae) parasitize later larval instars, and *Pseudorhyssa nigricornis* (also Ichneumonidae) is a kleptoparasitoid attacking the rhyssines (Foelker and Parry 2021) (Table 1). The biologies of these native parasitoid species are not well understood, although molecular analyses have shown that the rhyssines (analyzed as a group) and *I. l. ensiger* parasitize both *S. nigricornis* and *S. noctilio* (Foelker et al. 2016b).

The dimorphic nematode, *Deladenus siricidicola*, is also a parasite of *S. noctilio*. The impact of nematodes on host woodwasps is determined by both nematode and woodwasp species and genotypes (Bedding 1972). Different species and strains of *Deladenus* parasitizing differing species and strains of *Sirex* are known to either totally sterilize (i.e., kill all host eggs in adult females), partially sterilize, or not sterilize parasitized hosts (van Nouhuys et al. 2022). The INA (= Introduced to North America; formerly referred to as 'non-sterilizing') strain of *D. siricidicola* that was putatively accidentally introduced with *S. noctilio* to North America does not sterilize eggs of *S. noctilio* but causes a decrease in adult size and fecundity (Kroll et al. 2013; Hajek and Morris 2021). *Deladenus siricidicola* also occurs as a free-living mycophagous form that feeds on the *Sirex*-symbiotic fungus within infested pines. Several strains of *S. noctilio* have been introduced to North America from unknown sources and origins (Boissin et

al. 2012; Bittner et al. 2017), and the INA strain of *D. siricidicola* is also from an unknown source, probably within the native distribution of *S. noctilio* (Morris et al. 2020). A native dimorphic nematode in pines, *Deladenus proximus*, described originally as a parasite of *S. nigricornis* (Bedding 1974), is never (Kroll et al. 2013; Haavik et al. 2016) or only rarely (Morris et al. 2013) recorded parasitizing *S. noctilio*.

Invasive *S. noctilio* in northeastern North America is therefore attacked by numerous species of native parasitoids as well as an introduced parasitic nematode (Table 1). This woodwasp species did not co-evolve with these parasitoids and it is questionable whether *S. noctilio* in any tree might have co-evolved with the nematode strain within that tree. Regardless, these natural enemies occupy the same community and rely on the same host as a resource, with the parasitoids killing woodwasps as they develop and nematodes living as internal parasites in *S. noctilio* larvae and adults.

Our overall goal in this study was to investigate relations among the parasitic nematode, the native parasitoid community, and the invasive host woodwasp in naturally occurring attacked trees. We evaluated emergence from 204 *S. noctilio*-infested trees from multiple forested areas in northeastern North America between 2011 and 2019. We tested the hypotheses that *S. noctilio* aggregate within a few of the infested trees in a stand, and that the natural enemies would each respond positively to host density. Further, since the natural enemies co-occur but cannot all successfully infect the same host individuals, there would be negative associations among them where their densities were high enough for competition to occur. Given the patterns of tree infestation and parasitism that we found, we discuss likely consequences to populations of the native woodwasp *S. nigricornis* from invasion of this community by *S. noctilio*. Results from this study will improve our understanding of population level interactions in mixed invasive and native communities, as well as the ecology of this host/parasite community, as the invasive host continues to expand its range in North America.

Materials and methods

Between 2010 and 2018, 204 *S. noctilio*-infested pines were identified at sites where active infestations of *S. noctilio* were known or hypothesized to be present in pine forests at 21 sites in New York and Pennsylvania, USA (Table 2). Detection generally occurred from October to December. Nearly all of the pines were *Pinus resinosa*. Infested trees were patchily distributed within the forest stands. When searching for infested trees, trunks were inspected to 3 m for resin beads as these are indications of infestation (Haavik and Foelker 2021). In spring 2011–2019, before *S. noctilio* emergence, the trees that had been identified as infested were felled and areas of the trunks with resin beads were cut into 70 cm long bolts. Insects were reared from this wood and collected as they emerged, as described in Hajek et al. (2017). Throughout, the years presented for samples are the years in which emergence occurred.

Sirex and parasitoid adults that emerged were kept individually in vials at 4 °C. *Sirex noctilio* and *S. nigricornis* were identified using Schiff et al. (2006, 2012) and

Table 2. Localities of collections of *Sirex*-infested trees in New York state and Pennsylvania.

Year	State	County	Site*	*Pinus* species	GPS	No. trees
2011	New York	Schuyler	Arnot Forest	*P. sylvestris*	42.26445, -76.62757	2
2011	Pennsylvania	Tioga	TSF: Government Rd	*P. resinosa*	41.65038, -76.92572	9
2011	Pennsylvania	Tioga	TSF: Mountain Ridge Rd	*P. resinosa*	41.74501, -76.94507	6
2011	New York	Cortland	Heiberg Forest	*P. resinosa*	42.76094, -76.08341	2
2011	New York	Tompkins	Waterburg Rd	*P. resinosa*	42.49939, -76.67325	3
2011	New York	Broome	Triangle	*P. resinosa*	42.34022, -75.88022	1
2012	New York	Schuyler	Arnot Forest	*P. resinosa*	42.26445, -76.62757	9
2012	New York	Tompkins	Danby	*P. sylvestris*	42.37903, -76.47251	1
2012	New York	Steuben	Cameron State Forest	*P. resinosa*	42.26578, -77.41622	1
2012	New York	Schuyler	Finger Lakes National Forest	*P. resinosa*	42.47374, -76.77994	3
2012	New York	Warren	Nr. Pack Forest	*P. resinosa*	43.51550, -73.81478	6
2012	Pennsylvania	Tioga	TSF: Government Rd	*P. resinosa*	41.65038, -76.92572	14
2012	Pennsylvania	Tioga	Hills Creek State Park	*P. resinosa*	41.85348, -77.19989	11
2012	Pennsylvania	Tioga	TSF: Hypocrite Trail	*P. resinosa*	41.67096, -76.92317	18
2012	Pennsylvania	Tioga	Leonard Harrison State Park	*P. resinosa*	41.69646, -77.45460	5
2013	Pennsylvania	Tioga	TSF: Hypocrite Trail	*P. resinosa*	41.67096, -76.92317	12
2013	Pennsylvania	Tioga	Hills Creek State Park	*P. resinosa*	41.85348, -77.19989	5
2013	Pennsylvania	Tioga	Leonard Harrison State Park	*P. resinosa*	41.69646, -77.45460	2
2014	New York	Schuyler	Arnot Forest	*P. resinosa*	42.28194, -76.63138	2
2014	Pennsylvania	Tioga	Hills Creek State Park	*P. resinosa*	41.85348, -77.19989	26
2014	New York	Cortland	Hewitt State Forest	*P. resinosa*	42.74353, -76.22174	2
2015	Pennsylvania	Tioga	TSF: Arnot Forest	*P. resinosa*	41.67380, -77.14184	4
2015	New York	Schuyler	Arnot Forest	*P. resinosa*	42.28194, -76.63138	1
2015	Pennsylvania	Tioga	TSF: Fellows Creek	*P. resinosa*	41.69965, -76.96671	5
2015	Pennsylvania	Tioga	Hills Creek State Park	*P. resinosa*	41.85348, -77.19989	13
2015	Pennsylvania	Tioga	Ridge Road	*P. resinosa*	41.67589,-76.95881	1
2015	New York	Oneida	Sand Flats State Forest	*P. sylvestris*	43.55212, -75.27708	3
2015	Pennsylvania	Tioga	TSF: Tanglewood	*P. resinosa*	41.71145, -76.98356	4
2016	Pennsylvania	Tioga	TSF: Hypocrite Trail	*P. resinosa*	41.67096, -76.92317	3
2017	Pennsylvania	Clarion	Corsica	*P. sylvestris*	41.17809, -79.22653	10
2017	Pennsylvania	Indiana	Hillsdale	*P. resinosa*	40.75009, -78.88494	10
2017	Pennsylvania	Tioga	TSF: Hypocrite Trail	*P. resinosa*	41.67096, -76.92317	3
2019	Pennsylvania	Tioga	TSF: Government Rd.	*P. resinosa*	41.65038, -76.92572	3
2019	Pennsylvania	Tioga	TSF: Hypocrite Trail	*P. resinosa*	41.67096, -76.92317	4

*TSF = Tioga State Forest

associated parasitoids were identified using Standley et al. (2012). In 2011, we were unable to distinguish between some rhyssines, and the rhyssines emerging in 2012 were not all identified, so these are listed as *Rhyssa* spp. (Suppl. material 1).

To evaluate nematode parasitism, subsamples of *S. noctilio* from each site and year were dissected as described in van Nouhuys et al. (2022) and presence of parasitic nematodes within bodies was recorded. A total of 3154 randomly chosen *S. noctilio* were dissected to detect whether they were parasitized by nematodes. Species of nematodes were not evaluated using molecular methods for this study but, based on past results (Kroll et al. 2013; Williams and Hajek 2017), we presume that the vast majority

of parasitic nematodes were *D. siricidicola* INA. Another nematode species, *Deladenus proximus*, is primarily known from *S. nigricornis* hosts. *Deladenus proximus* was reported parasitizing a few *S. noctilio* collected in New York state in 2010 (Morris et al. 2013). However, *D. proximus* are usually found in the host eggs (Hartshorn 2021; van Nouhuys et al. 2022), and in our study nematodes were only seen within eggs from 4 *S. noctilio* from 2 sites in 2011. This was the first year of this study and there *S. nigricornis* were more abundant (Suppl. material 1).

Data analysis

Sirex density was quantified as all of the emerged *Sirex* plus each emerging *Sirex* parasitoid, as each parasitoid is solitary, representing one host individual. The native woodwasp *S. nigricornis* emerged from 25 of the 204 trees in five of the eight years. From 22 of these trees, *S. noctilio* emerged as well, usually in much higher quantities than *S. nigricornis*. From the remaining three trees only *S. nigricornis* emerged. As *S. noctilio* had emerged from other trees from those sites sampled throughout the same years and the parasitoids use both *Sirex* species as hosts (Foelker and Parry 2021), these trees were included in the study. *Sirex nigricornis* individuals that emerged were included in the *Sirex* density estimates because they are hosts to both the parasitoids and the nematode (Morris et al. 2013). For the statistical analyses involving the rhyssines all five species were combined because no one species was common enough for separate analysis, and because in some cases the individual species were not differentiated (Suppl. material 1).

All statistical analyses were done using JMP (SAS Institute Inc. 2021). For the analysis of aggregation of *Sirex* among trees we compared the distribution of samples among trees within a site with a Poisson distribution which is what is expected if the wasps were independent and equally likely to emerge from any of the sampled trees. The statistical comparison among counts per tree was done using a Poisson dispersion test (Spinelli and Stephens 1997) for the 12 sites in which 6 or more trees were sampled within a year. Sites with fewer trees provided too little data on tree-to-tree distribution to meaningfully conduct such tests.

For the analysis of the association of nematode infection with per tree *Sirex* density and rate of parasitism by parasitoids we used logistic regression. The response variable was the presence/absence of nematodes in each of the 3154 *S. noctilio* dissected to detect nematodes. The samples collected from 2012 were not included because very few *S. noctilio* were dissected that year. The explanatory variables were year, site nested in year where sites were sampled across multiple years, the log of the number of *Sirex* in the tree the sample came from, the fraction of hosts parasitized by *I. l. ensiger* and by the combined rhyssine parasitoids in that tree, and interactions between the host and each parasitoid, and between *I. l. ensiger* and the combined rhyssines. Interactions that did not contribute to the model were removed. Because the number of wasps (*Sirex* + parasitoids) per tree ranged widely (from 1 to 864), with many trees occupied by few wasps, the number of wasps was log-transformed. Because the different parasitoids

may have different patterns of parasitism and potential associations with the nematode, we separated *I. l. ensiger* and the combined rhyssine parasitoids.

For the analysis of association of parasitism by parasitoids with woodwasp density at the tree level, we used logistic regression with the full data set of all 204 trees that wasps emerged from over all eight years. Three separate models were tested. The binomial response variable in the first was whether a sample was a parasitoid or *Sirex*. In the second model the response variable was whether the sample was *I. l. ensiger* or not (if not *I. l. ensiger* it could be a *Sirex* or a rhyssine). In the third model the response variable was whether the sample was a rhyssine or not (if not a rhyssine it could be *Sirex* or *I. l. ensiger*). The explanatory variables in all three models included year, site nested in year, and the log of the number of *Sirex* in the tree the sample came from (host density). Since each parasitoid can have an independent relationship to host density, in the model for total parasitism the per tree rates of parasitism by *I. l. ensiger* and rhyssines, as well as their interactions with host density, were included as explanatory variables. The model for *I. l. ensiger* included the per tree rate of parasitism by rhyssines, and its interaction with host density as response variables. The model for rhyssines included per tree rate of parasitism by *I. l. ensiger*, as well as the interaction of *I. l. ensiger* parasitism and host density as response variables.

Results

Between 2011 and 2019, *S. noctilio* and their parasitoids were reared from 204 *S. noctilio*-infested trees from Pennsylvania and New York State (Table 2). There were 9852 adult woodwasps or parasitoids that emerged over the eight years of sampling. Of these, 3870 were parasitoids, 5804 were *S. noctilio*, and 178 were the native woodwasp *S. nigricornis* (Suppl. material 1). Most trees (130 of 204, 63.7%) were infested with 1–25 *Sirex* (adult *Sirex* + their parasitoids). However, 45.7% of the population occupied 12 of 204 trees. These trees each contained more than 198 *S. noctilio*, with 854 individuals emerging from the tree with the highest occupancy (Fig. 1). Thus, the distribution of *Sirex* equivalents (adult *Sirex* + their parasitoids) among trees was strongly aggregated, differing significantly from the expected Poisson distribution that would occur if they were distributed randomly among infested trees. This is true for the combined data (χ^2 = 1700.34; $P < 0.0001$) (Fig. 1), as well as in each of 12 sites analyzed separately (χ^2 ranged from ≈ 0.00 to χ^2 = 121.34, all are $P < 0.0001$). The distributions of individuals among trees in the six sites with the highest densities are presented in Fig. 2.

Overall, 39% of the *Sirex* were parasitized by parasitoids. The most abundant parasitoid species was *I. l. ensiger*, which comprised 81% of the parasitoids reared and was found at every site. Next most abundant was *Rhyssa lineolata*, which made up 63% of the rhyssines (leaving out samples from 2012 when rhyssine species were not separated, Suppl. material 1) and was also found at every site, although densities were always less than *I. l. ensiger*. The other four rhyssines (two *Rhyssa* species: *R. persuasoria* and

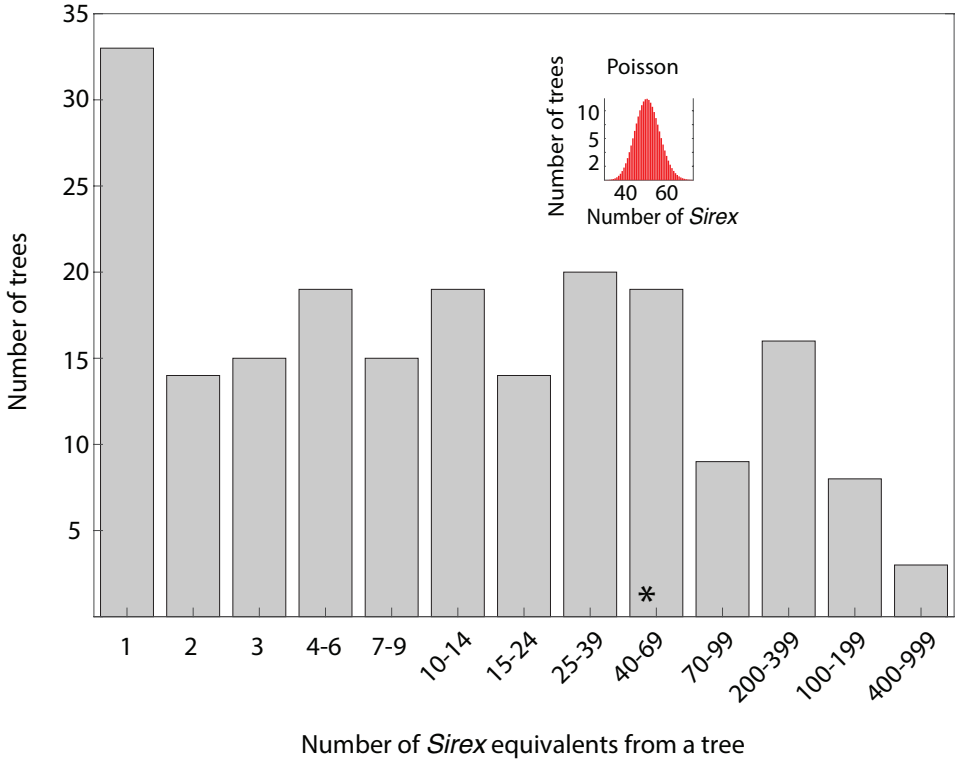

Figure 1. The distribution of *Sirex* equivalents (*Sirex* adults + parasitoid adults) emerging from trees in the whole data set. The inset shows the distribution of *Sirex* equivalents among the same number of trees if the *Sirex* were distributed randomly among trees (a Poisson distribution). The * indicates the mean number of wasps per tree (48.3). The number of wasps along the x-axis is shown in a modified log scale in order to make both small and large numbers of wasps per tree visible.

R. crevieri, *Megarhyssa nortoni*, and the kleptoparasitoid *Pseudorhyssa nigricornis*) occurred much less commonly. The 8 collected *M. nortoni* came from three sites in the years 2011 and 2013. Most of the 13 *P. nigricornis* came from Hypocrite Trail in Tioga State Forest (Pennsylvania), in two different years (Suppl. material 1).

Nematodes were found in 11% of the dissected *S. noctilio*. The proportion of nematode-parasitized hosts in a tree was positively associated with host density (χ^2 = 4.92; P = 0.0265; Table 3a; Fig. 3a). There was no association with parasitism by *I. l. ensiger* (χ^2 = 0.004; P = 0.9460; Table 3a), but a negative association occurred with parasitism by the rhyssine parasitoids (χ^2 = 31.57; P < 0.0001; Table 3a; Fig. 3b). This was especially true at low host densities ($\chi^2_{rhyssines \times host\ density}$ = 21.67; P = 0.0001; Table 3a). Nematode parasitism also differed between years (χ^2 = 166.04; P < 0.0001; Table 3a), as well as among sites within years (χ^2 =329.13; P < 0.0001; Table 3a). For instance, only 7% of hosts dissected from the Corsica and Hillsdale, PA sites sampled in 2017

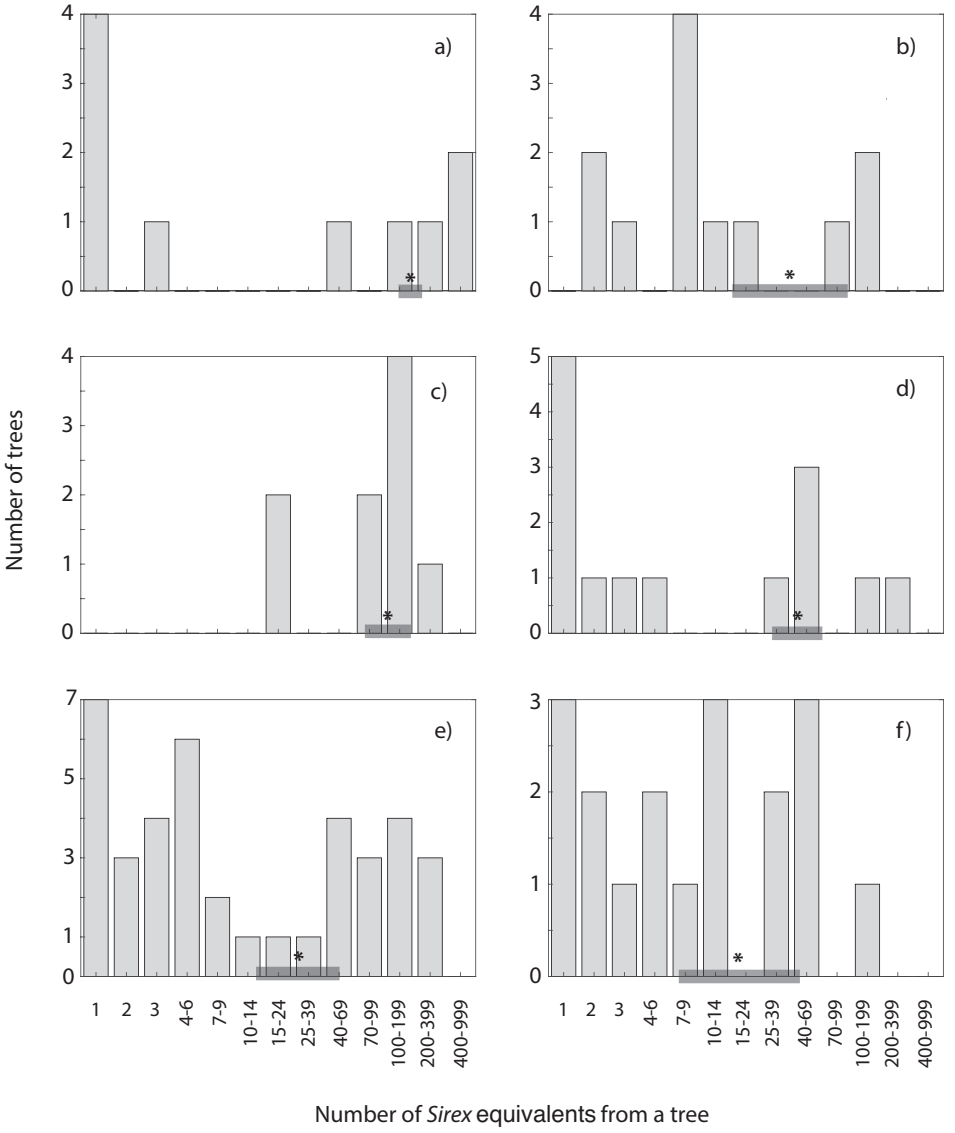

Figure 2. The distribution of *Sirex* equivalents (*Sirex* adults + parasitoid adults) emerging from the 6 sites with the most data (each had 398 or more *Sirex* equivalents and nine or more trees). The wasps were strongly aggregated in these and the other 6 sites tested (see text). * denotes the mean number of *Sirex* equivalents per tree. The horizontal gray bars represent the 95% CI range of wasps per tree if the *Sirex* were distributed randomly among the trees, which would be a Poisson distribution (as shown in the inset in Fig. 1). The number of wasps along the x-axis is shown in a modified log scale to make both small and large numbers of wasps visible. a) 2017 Hillsdale site, 2052 *Sirex* equivalents from 10 trees, b) 2013 Hypocrite Trail site, 400 *Sirex* equivalents from 12 trees, c) 2011 Government Rd. site, 1058 *Sirex* equivalents from 9 trees, D) 2012 Government Rd. site, 575 *Sirex* equivalents from 14 trees, e) 2014 Hypocrite Trail site, 2055 *Sirex* equivalents from 39 trees, f) 2012 Hypocrite Trail site, 398 *Sirex* equivalents from 18 trees.

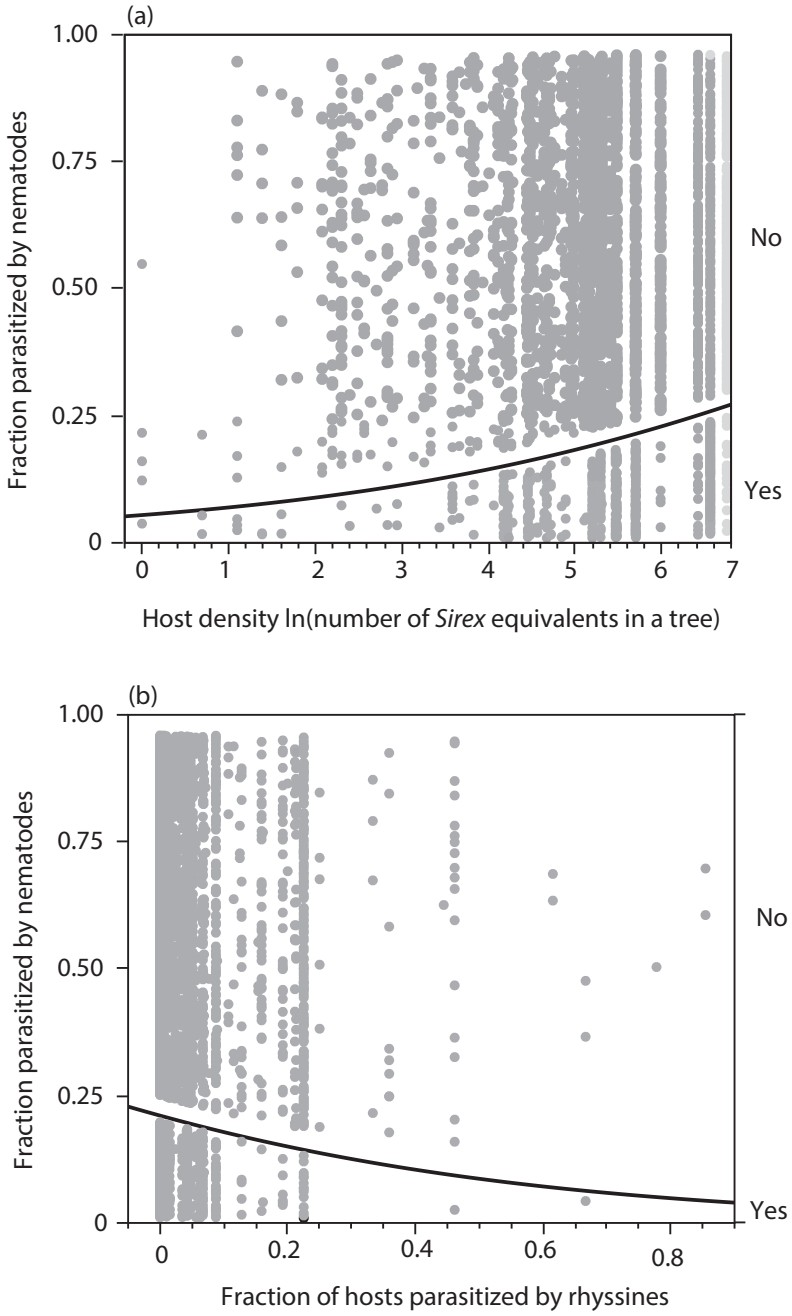

Figure 3. The association of parasitism by the nematode *Deladenus siricidicola* with **a** per tree *Sirex* density (Logistic regression model coefficient $P < 0.026$; Table 3a) and **b** fraction of *Sirex* in the tree parasitized by rhyssines (Logistic regression model coefficient $P < 0.0001$; Table 3c). Each point represents a dissected *S. noctilio*. The points are distributed vertically in order to show the amount of data at each host density. The points below the curves are parasitized *D. siricidicola* (Yes) and the points above the curve are not (No). See the text for explanation of the statistical models.

Table 3. Results of the logistic regression analyses of a) nematode parasitism, and parasitism by b) all parasitoids, c) *I. I. ensiger*, and d) rhyssines. See text for details of models.

Parameter	df	Estimate(SE)	χ^2	$P > \chi^2$	Odds ratio	Effects likelihood ratio χ^2	$P > \chi^2$
a) Nematodes							
Year	6	multiple				166.04	<0.0001
Site (Year)	20	multiple				329.13	<0.0001
Host density*	1	0.23(0.10)	4.72	0.0298	1.26	4.92	0.0265
Rhyssines rate**	1	-5.81(1.14)	26.15	<0.0001	0.01	31.57	<0.0001
Rhyssines rate × host density	1	5.05(1.20)	17.66	<0.0001		21.67	<0.0001
I. I. ensiger rate	1	-0.04(0.58)	0.00	0.9460	0.97	0.004	0.9460
b) All parasitoid wasps							
Year	7	multiple				1.25	0.9898
Site (Year)	26	multiple				13.48	0.9793
Host density	1	-0.002(0.03)	0.00	0.9522	0.99	0.003	0.9522
I. I. ensiger rate	1	4.62(0.26)	321.88	<0.0001	101.72	348.39	<0.0001
I. I. ensiger rate × host density	1	-0.29(0.16)	3.21	0.0734		3.34	0.0674
Rhyssines rate	1	4.65(0.26)	283.23	<0.0001	105.37	316.39	<0.0001
Rhyssines rate × *I. I. ensiger* rate	1	-3.29(1.31)	6.30	0.0121		6.46	0.0110
c) *I. I. ensiger*							
Year	7	multiple				17.68	0.0135
Site (Year)	26	multiple				211.78	<0.0001
Host density	1	0.18(0.03)	30.51	<0.0001	1.19	31.88	<0.0001
Rhyssines rate	1	0.42(0.24)	3.07	0.0797	1.51	3.04	0.0813
Rhyssines rate × host density	1	1.16(0.16)	53.44	<0.0001		62.39	<0.0001
d) Rhyssines							
Year	7	multiple				422.98	<0.0001
Site (Year)	26	multiple				256.20	<0.0001
Host density	1	-0.22(0.59)	13.52	0.0002	0.80	12.60	0.0004
I. I. ensiger rate	1	0.71(0.41)	3.00	0.0830	2.04	3.00	0.0832
I. I. ensiger rate × host density	1	1.92(0.23)	72.32	<0.0001		82.44	<0.0001

*For each statistical model Host density is the log of the number of *Sirex* equivalents emerging from a tree.
** For each statistical model Rate is the fraction of *Sirex* parasitized by *I. I. ensiger* or Rhyssines within a tree.

were parasitized by nematodes, whereas 82% of hosts dissected at the Tanglewood, PA site in 2015 were parasitized by nematodes (Fig. 4).

Overall parasitism by parasitoids was unrelated to tree-level host density ($\chi^2 = 0.003$; $P = 0.9522$; Table 3b). However, parasitism by *I. l. ensiger* increased with host density ($\chi^2 = 31.89$; $P < 0.0001$; Table 3c; Fig. 5a), while parasitism by the rhyssines decreased with host density ($\chi^2 = 12.60$; $P = 0.0004$; Table 3d; Fig. 5b). Parasitism by *I. I. ensiger* had no overall association with rate of parasitism by the rhyssines ($\chi^2 = 3.04$; $P = 0.0813$; Table 3c), except at low host densities where rhyssine parasitism was high ($\chi^2_{rhyssines \times ln(host)} = 62.39$; $P = < 0.0001$; Table 3c). Similarly, parasitism by the rhyssines also had no overall association with rate of parasitism by *I. l. ensiger* ($\chi^2 = 3.00$; $P = 0.0832$; Table 3d), except at low host density ($\chi^2_{I.\ l.\ ensiger \times ln(host)} = 82.44$; $P = < 0.0001$; Table 3d). Parasitism by *I. l. ensiger*, rhyssines, and total parasitism each differed significantly among years and among sites (Table 3b, c, d).

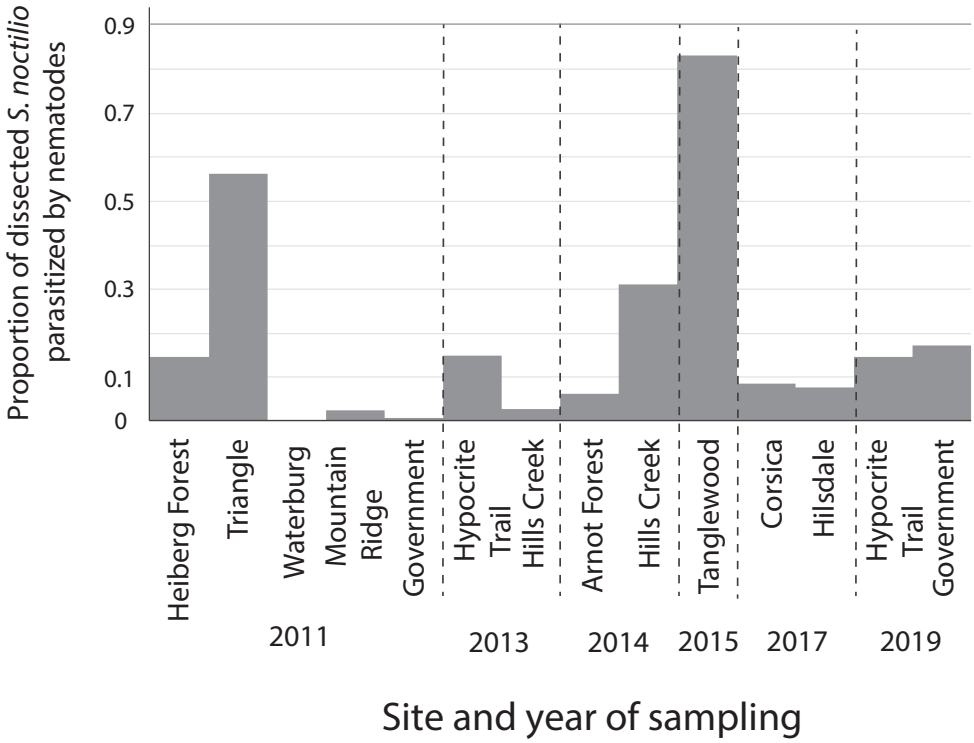

Figure 4. The proportions of dissected *S. noctilio* parasitized by *D. siricidicola* at each site between 2011 and 2019. Sites with fewer than 10 dissected *S. noctilio* are excluded as well as all sites from 2012.

Discussion

The invading *S. noctilio* exhibited very strong aggregation in specific trees within a stand, with up to 854 *Sirex* equivalents emerging from one tree and few *S. noctilio* emerging from the majority of infested trees (Figs 1, 2). In North America, Krivak-Tetley et al. (2022) and Haavik et al. (2018) reported a similar pattern of higher densities in few trees with lower densities in most. In Galicia, Spain, where *S. noctilio* is native, the pattern of aggregated emergence also occurred in pine plantations although the overall population was lower and the maximum emerging from one tree was 50 *S. noctilio* (Lombardero et al. 2016).

Generally, aggregated resource use can result from variation of resource quality (Woodcock et al. 2002), host susceptibility (Poulin 2013), or behavioral attraction to conspecifics (Cronin and Strong 1999). In this case, woodwasps are often attracted to weakened pines that are more susceptible to attack (Haavik and Foelker 2021); the occurrence of weakened trees can be a limiting and ephemeral resource for woodwasps. Additionally, *S. noctilio* females are attracted to the odor of the *Sirex*-associated fungal symbiont (Sarvary et al. 2015; Faal et al. 2021). Thus, *S. noctilio* may be strongly attracted to trees with many *Sirex* because those trees would already have been colonized by the *Sirex*-symbiotic fungus.

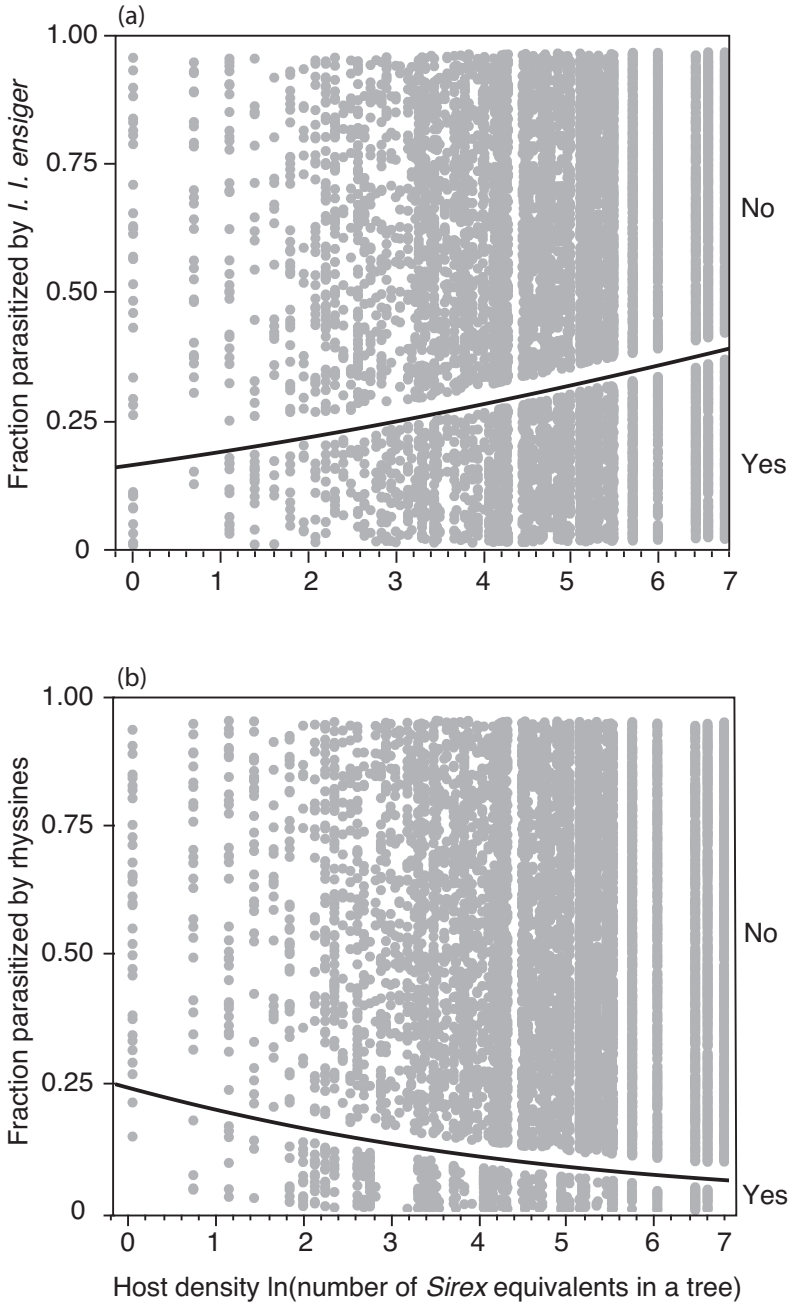

Figure 5. The association of parasitism by parasitoids with tree level host density. The curves show the logistic fit for **a** *I. l. ensiger* ($P < 0.0001$; Table 3c) and **b** rhyssines ($P = 0.0004$; Table 3d). The horizontal axis shows host density as the log of the number of *Sirex* equivalents (adult *Sirex* + all parasitoids) emerging from a tree. Each data point is one sample. The points are distributed vertically in order to show the amount of data at each host density. The points below the curve are parasitoids (Yes) **a** *I. l. ensiger* and **b** rhyssines, and the points above the curve are unparasitized *Sirex* (No). See text for explanation of the statistical models.

The nematode *D. siricidicola* was present in 11% of the *S. noctilio* which is less than reports from many smaller studies in North America (see Krivak-Tetley et al. 2022), though nematode parasitism levels were variable and were much higher in some sites (Fig. 4). The fraction of samples in a tree that were parasitized by *D. siricidicola* increased strongly with host density (Fig. 3a). This is in contrast to the negative association found by Kroll et al. (2013) when studying fewer trees (n = 25) over one year. Positive host density dependence could result from a higher chance of nematode-infected females ovipositing in the same trees as healthy females at high density. Additionally, spread of the nematode among woodwasps within trees would be facilitated by high host densities. One reason for this is that high woodwasp density would result in high densities of the fungus *A. areolatum*, which is eaten by these nematodes when they are mycophagous. More *A. areolatum* throughout trees would potentially facilitate greater dispersal by the mycophagous-phase nematodes within trees, which would result in more nematodes reaching and parasitizing *Sirex* larvae.

Nematodes may parasitize a woodwasp before or after it has been parasitized by a wasp. Once within a shared host, nematodes could, in principle, move from the woodwasp larva to a parasitoid larva. However, the nematode *D. siricidicola* is specific to *Sirex* as well as parasitizing a wood-dwelling genus of beetle associated with siricids (*Serropalpus*) (Bedding and Akhurst 1978; Bittner et al. 2016). Other species of parasitic nematodes have been reported from rhyssines in other locales (Bedding and Akhurst 1978). However, Morris et al. (2020) evaluated 388 adult rhyssines emerging from pines between 2012–2015 in the same research area that we studied and found no nematodes in them. Thus, we do not believe that *D. siricidicola* in this study were parasitizing the parasitoids.

Further, the nematode *D. siricidicola* cannot successfully develop in or be vectored by a *Sirex* parasitized by a parasitoid because the host is killed by the parasitoid. While the nematodes would not succeed in such a shared host, parasitoids likely can develop in *Sirex* larvae that are also parasitized by nematodes. There may be a cost to parasitoids due to reduced host size since nematode-parasitized *Sirex* are somewhat smaller than unparasitized individuals (Hajek and Morris 2021). Additionally, nematode infection has been shown in some systems to increase host immune response, including parasite encapsulation (Castillo et al. 2011), which could reduce the success of parasitoids. Alternatively, immune suppression by the nematode combined with immune suppression due to the parasitoid venom could be detrimental to the host, causing it to die or to have greater susceptibility to other parasites (Shaik et al. 2020). Nonetheless, we expect that when the nematode *D. siricidicola* and a parasitoid are using the same host individual, the parasitoid would be the superior competitor. We found that parasitism by nematodes was neither positively nor negatively associated with parasitism by the abundant parasitoid *I. l. ensiger* (Fig. 3b), but it was negatively associated with parasitism by the rhyssines (Fig. 3c).

The observed negative association of nematode parasitism with rhyssines may simply be because the nematodes are strongly positively host density dependent, so they are nearly absent from trees with low host density, which are the ones in which

parasitism by rhyssines was highest. Alternatively, competition between nematodes and rhyssines at low host densities may decrease the number of nematode-infected *Sirex*, contributing to the pattern of host density dependence of the nematodes. While such competition may contribute to the pattern, it is not the full explanation because at low host density nematode parasitism is near 0 (Fig. 3a), and rhyssine parasitism reaches only 24% (Fig. 5b).

Both the nematode and the abundant parasitoid *I. l. ensiger* increase with host density within trees. Yet there is no evidence of direct competition, since even accounting for host density, high *I. l. ensiger* density was not associated with lower prevalence of nematodes. This suggests that there could be some spatial segregation of host use. For example, *I. l. ensiger*, which have short ovipositors, have been found to be most abundant high up in trees where bark is thinner (Foelker et al. 2016a; Long et al. 2009). Studies in South Africa found that the wood at the bases of pines was moister than that higher within the trunks, and *D. siricidicola* densities were also greater at the bases of trees; numerous studies have suggested that wood moisture content can influence activity of both *D. siricidicola* and *A. areolatum* (see Hurley et al. 2008). If the nematodes are less abundant higher in trees due to such an environmental variable, then direct competition with *I. l. ensiger* would be reduced. Alternatively, nematode mortality due to competition with the parasitoid could be masked by very strong host density dependence by the nematodes.

The native community of parasitoids that were present in the northeastern US before invasion by *S. noctilio* readily use *S. noctilio* as a host. On average, 37% of the *Sirex* from a tree was parasitized, ranging from 0 to 100%. This overall parasitism is similar to what was found by Krivak-Tetley et al. (2022) in a smaller study in this region, and by Haavik et al. (2015) using experimentally-exposed logs. We found the frequency of parasitism by *I. l. ensiger* increased with host density, and the frequency of parasitism by rhyssines decreased with increasing host density, leading to no association of overall parasitism with host density (Table 3). Different parasitoid species often respond differently to host density. Some species respond to high host density by aggregating, which leads to a positive dependent rate of parasitism (Walde and Murdoch 1988; Ives 1992). Indeed, *I. l. ensiger* has been shown to be attracted to the fungus associated with *S. noctilio* (Martinez et al. 2006; Faal et al. 2021) and to increase residence time on trees at increased *S. noctilio* density (Corley et al. 2010). However, in this complicated system, Foelker et al. (2016a) found no association of rate of parasitism by *I. l. ensiger* with host density while Krivak-Tetley et al. (2022) found a host density association for *I. l. ensiger* in *Pinus resinosa*, the pine predominantly sampled in this study, but not in *Pinus sylvestris*. Such differences between studies are not surprising where there is density dependence, especially when studies are conducted at different host densities. Our study was conducted over a wide range of host densities.

The rhyssine species showed higher parasitism at low host density. If parasitoids do not respond to host density the fraction parasitized can appear higher at very low host density than at high host density, simply reflecting the population size of foraging females (Walde and Murdoch 1988). This same pattern could also be due to competi-

tion between the rhyssines and *I. l. ensiger*. The latter species parasitizes *Sirex* eggs or young larvae (Foelker and Parry 2021) which makes it likely to be the superior competitor, since later-arriving endoparasitoids rarely are able to kill the parasitoid already developing in a host (van Nouhuys and Punju 2010; Cusumano et al. 2016). We only found a significant negative association between the parasitoid wasps at low host densities, where the fraction of hosts parasitized by rhyssines was highest. At higher host densities the frequency of parasitism by rhyssines was low, so the chance of both species parasitizing the same host individuals was unlikely, especially if there is some spatial segregation of parasitoid species within trees (Long et al. 2009; Foelker et al. 2016a).

As a caveat, the interactions between parasitoids and *S. noctilio* is complicated to evaluate due to variable voltinism of both rhyssines and *S. noctilio* (Foelker et al. 2016a). In particular, rhyssines have been thought to oviposit on late instar siricids in spring but then only emerge the following spring (Foelker and Parry 2021). However, it has also been suggested that two generations of rhyssines could possibly occur per year (Foelker and Parry 2021), which is supported by findings in this study, in which rhyssines emerged from the same wood in the same year as *S. noctilio*. In addition, there is the complication that *S. noctilio* has been found to oviposit in the same trees over multiple years (Haavik et al. 2018) and, once developing within a tree, some of the *S. noctilio* require more than one year for development (Ryan et al. 2012; Myers et al. 2014; Hajek et al. 2017).

In this study the native woodwasp, *S. nigricornis* occurred at much lower densities than *S. noctilio*, with the majority emerging early in the study, between 2011 and 2014 (Suppl. material 1); this supports the hypothesis that *S. noctilio* is the more successful competitor (Hajek et al. 2017). Of the 26 trees from which *S. nigricornis* emerged, *S. noctilio* also emerged from 88.5% of them. The local high densities of *S. noctilio* can impact the native woodwasp *S. nigricornis* in several ways. The large populations of *S. noctilio* could directly compete with *S. nigricornis* for trees in which to oviposit, especially because *S. nigricornis* is considered a less aggressive colonizer (i.e., needing weaker trees than *S. noctilio*) and most oviposit after *S. noctilio* (Hartshorn 2021). Secondly, when both woodwasps occupy the same tree, which creates higher woodwasp densities, parasitism by the nematode and *I. l. ensiger*, which are positively host density dependent, would be higher than if *S. nigricornis* occurred alone (van Nouhuys and Kraft 2012). Finally, high *S. noctilio* population densities can drive up the shared parasitoid and nematode populations in a forest, which could cause *S. nigricornis* to decline, an example of spillback by an invasive species (Kelly et al. 2009). Such apparent competition, driven by shared natural enemies, can have a strong impact on insect communities (van Nouhuys and Hanski 2000; Holt and Bonsall 2017).

In summary, this study of invasive *S. noctilio* and its introduced and native natural enemies emerging from 204 naturally infested trees over eight years, allowed for a robust analysis of the distribution of *S. noctilio* among trees, and for an exploration of the complex association of host and natural enemies over a large range of natural host densities. We found a strong pattern of aggregation by the woodwasp in a subset of the trees it occupied. Parasitism by the nematode *D. siricidicola* increased with host

density, making its distribution also aggregated. Parasitism by the native woodwasp parasitoid *I. l. ensiger* was high and also increased with host density, suggesting that this wasp may contribute to the control of the woodwasp. Additionally, though both the nematode and *I. l. ensiger* were positively host density dependent, there was little evidence of direct competition between the parasitoid and the nematode. The suite of native rhyssine parasitoids of *Sirex* were less abundant and occurred mostly where host densities were low. While our study indicates that they suffer from competition with *I. l. ensiger*, their population level relationship with the host and the other parasitoids may be complicated by a variation of voltinism.

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Supplementary material 1

The number of *Sirex*, nematodes, and parasitoids collected from each site, each year.

Authors: Saskya van Nouhuys, David C. Harris, Ann E. Hajek

Data type: Occurance

Explanation note: Data table showing the number of individuals of each species collected as part of this study.

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RESEARCH ARTICLE

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Genetic analyses reveal a complex introduction history of the globally invasive tree *Acacia longifolia*

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Abstract

Acacia longifolia (Sydney golden wattle) is considered one of the most problematic plant invaders in Mediterranean-type ecosystems. In this study, we investigate the species' invasion history by comparing the genetic diversity and structure of native (Australia) and several invasive range (Brazil, Portugal, South Africa, Spain, and Uruguay) populations and by modelling different introduction scenarios using these data. We sampled 272 *A. longifolia* individuals – 126 from different invasive ranges and 146 from the native range – from 41 populations. We genotyped all individuals at four chloroplast and 12 nuclear microsatellite markers. From these data we calculated diversity metrics, identified chloroplast haplotypes, and estimated population genetic structure based on Bayesian assignment tests. We used Approximate Bayesian Computation (ABC) models to infer the likely introduction history into each invaded country. In Australia, population genetic structure of *A. longifolia* appears to be strongly shaped by the Bass Strait and we identified two genetic clusters largely corresponding to mainland Australian and Tasmanian populations. We found invasive populations to represent a mixture of these clusters. Similar levels of genetic diversity were present in native and invasive ranges, indicating that invasive populations did not go through a genetic bottleneck. Bayesian assignment tests and chloroplast haplotype frequencies further suggested a secondary introduction event between South Africa and Portugal. However, ABC analyses could not confidently identify the native source(s) of invasive populations in these two countries, probably due to the known high propagule pressure that accompanied these introductions. ABC analyses identified Tasmania as the likely source of invasive populations in Brazil and Uruguay. A definitive native source for Spanish populations could also not be identified. This study shows that tracing the introduction history of

A. longifolia is difficult, most likely because of the complexity associated with the extensive movement of the species around the world. Our findings should be considered when planning management and control efforts, such as biological control, in some invaded regions.

Keywords
Australian acacias, genetic diversity, haplotypes, introduction history, microsatellite markers, multiple introductions, population structure, propagule pressure

Introduction

Australian acacias (genus *Acacia* Mill.) are considered some of the world's most problematic plant invaders (Richardson et al. 2011). At least 25 species are known to be invasive (Richardson et al. 2015; Magona et al. 2018) and are amongst some of the best studied taxa in invasion biology (Richardson et al. 2011). *Acacia* species have been introduced worldwide for several purposes, such as land reclamation, tannin production and as ornamental plants (Griffin et al. 2011). These introductions frequently involved high propagule pressure (i.e., the number and size of introduction events), exemplified by invasive populations that often have high levels of genetic diversity and that experience no molecular inbreeding (Vicente et al. 2021).

Knowledge of the introduction history of invasive species may aid their management, such as biological control (Jourdan et al. 2019). For example, the earleaf acacia, *Acacia auriculiformis*, is considered highly invasive in Florida, USA (Kull et al. 2008; Richardson and Rejmánek 2011; McCulloch et al. 2021). In its native range, the species shows deep phylogenomic divergence between northern Queensland and the Northern Territory in Australia, and Papua New Guinea (McCulloch et al. 2021). This deep genetic divergence is mirrored in populations of some herbivorous insects feeding on *A. auriculiformis*, such as the specialist leaf-feeding beetle, *Calomela intemerata* (Nawaz et al. 2021). This holds important implications for the utility of this insect as a potential biocontrol agent for *A. auriculiformis*. For example, genomic analyses identified the Northern Territory as the source of invasive populations of the wattle in Florida (McCulloch et al. 2021) and, therefore, *Calomela intemerata* from this part of Australia may be particularly well-suited for biocontrol release in this invaded region.

Molecular studies have been instrumental in disentangling the introduction histories of invasive species (Le Roux 2021). For example, genetic studies allow researchers to trace the routes of, and estimate the propagule pressure that founded, invasions (Wardill et al. 2005; Pyšek et al. 2013). Invasive Australian acacias have been particularly well-studied in this regard (e.g., Thompson et al. 2012, 2015; Le Roux et al. 2013; Ndlovu et al. 2013; Vicente et al. 2018; Hirsch et al. 2019, 2021). For instance, the *Acacia saligna* species complex (also known as Coojong) comprises three distinct genetic lineages (ssp. *lindleyi*, ssp. *stolonifera*, and ssp. *salinga* and *pruinescens*; Millar et al. 2011) with different distributions in Australia (Thompson et al. 2011). This species was introduced into South Africa in 1833 (Poynton 2009), Portugal in 1869

(Thompson et al. 2015), Libya and Ethiopia in 1870 (Griffin et al. 2011), and Israel in 1920 (Kull et al. 2011). It was also introduced into countries such as Italy and the USA (California), where introduction dates are unknown (Thompson et al. 2015). Using genetic data, Thompson et al. (2012, 2015) found that all lineages have been introduced outside Australia and that extensive admixture is occurring in some invaded ranges. However, in South Africa a novel genetic lineage (which likely originated from an introgressive hybridisation event) was identified (Thompson et al. 2012). This lineage was subsequently identified in Italy and Portugal (Thompson et al. 2015). *Acacia pycnantha*, also known as the Golden wattle, is a species native to eastern and southern Australian that has two recognised ecotypes, the so-called wetland and dryland forms (Ndlovu et al. 2013). This species was introduced into South Africa in 1865 and again in 1890 and is now considered highly invasive in the country (Poynton 2009). The species also has invasive populations in Portugal and Western Australia (Richardson and Rejmánek 2011; Ndlovu et al. 2013). In Australia, Le Roux et al. (2013) identified admixture between the two ecotypes, probably because of the extensive movement of seeds/plants for restoration. Invasive populations in South Africa have similar levels of genetic diversity and admixture to native range populations (Le Roux et al. 2013).

Acacia longifolia (Andrews) Willd. is native to south-eastern Australia and Tasmania, with two formally described subspecies: *A. l.* ssp. *longifolia* and *A. l.* ssp. *sophorae* (Flora of Australia Volume 11B, Mimosaceae, *Acacia* part 2 2001). These subspecies are distinguished by phyllode shape, size and colour, and seed pod shape. They also have slightly different, but mostly overlapping, distributions in Australia (Flora of Australia Volume 11B, Mimosaceae, *Acacia* part 2 2001). This species has been introduced into several countries as an ornamental tree and for coastal dune stabilisation and is now considered one of the worst plant invaders in many Mediterranean regions. *Acacia longifolia* was initially introduced into South Africa in 1827. Subsequent introductions occurred in 1845 (from Australia) and between 1895 and 1908 (secondarily from Paris and California, and the Botanical Gardens of Adelaide; Poynton 2009). In 1984, *A. longifolia* was considered the second most impactful invader of the country's hyperdiverse fynbos biome (Macdonald and Jarman 1984; Dennill 1987). Between 1982 and 1983, the Australian gall-forming wasp, *Trichilogaster acaciaelongifoliae*, was introduced from Australia to control the species (Dennill 1985, 1987; Neser 1985). This biocontrol agent is considered highly successful (Janion-Scheepers and Griffiths 2020).

In Portugal, specimens of *A. longifolia* were recorded in the catalogue of the University of Coimbra's Botanical Gardens in 1878 (Henriques 1879). However, the first official introduction record of the species into Portugal likely dates to 1897 (Fernandes 2008; Carruthers et al. 2011; Kull et al. 2011). Irrespective of when the species was introduced, the origins of these introduction(s) remain unknown. The species was used by forestry services in several coastal afforestation projects over the years. A few examples are the dunes in São Jacinto (1888–1929; Marchante 2011), Costa da Caparica (1906; Lourenço 2009), Quiaios-Mira (1924 and 1948; Rei 1925; Marchante 2001, 2011), and Vila Nova de Milfontes [late 1960s/early 1970s; Miguel Prado, personal communication, but see Vicente (2016); Vicente et al. (2018)]. A molecular study

including three invasive populations from Portugal found similar genetic diversity and very low differentiation among them, which led to the hypothesis that the introduction of the species into Portugal involved a single seed source that was used to establish nursery stock, and these plants were then disseminated for afforestation projects (Vicente et al. 2018). However, the introduction records mentioned above suggest a more complex introduction history of the species into Portugal. The invasion success of *A. longifolia* in Portugal has led to the biological control release of *Trichilogaster acaciaelongifoliae*, imported from South Africa (Marchante et al. 2011). The wasp has successfully established along the Portuguese coast (López-Núñez et al. 2021).

In South America the introduction of *A. longifolia* occurred much more recently, in mid-20th century, into southern Brazil (Rico-Arce 2007; Zenni and Ziller 2011), Uruguay (Boelcke 1946; Rico-Arce 2007) and Argentina (Boelcke 1946; Celsi 2016). In Uruguay the first record for this species dates to 1946 (Boelcke 1946), and in Brazil the first official record of the species is from 1979 in Santa Catarina (Burkart 1979). There are also several observational records for the species in other states of Brazil, Argentina, and Uruguay (e.g., Base de dados de espécies exóticas invasoras do Brasil, http://bd.institutohorus.org.br; Tropicos.org, http://www.tropicos.org/Name/13024183). No biological control agents have been released into South America and no information is available on the origins of *A. longifolia* introductions to the continent.

Here we compare the population genetic diversity and structure of native and globally invasive populations of *A. longifolia*. We also aim to infer the introduction histories of the species into Brazil, Portugal, South Africa, Spain, and Uruguay using Approximate Bayesian Computation (ABC) modelling. Based on available historical records and the results from molecular studies of invasive acacias mentioned above, we hypothesised that the genetic diversity in invasive populations will be comparable to that of Australian populations, but with low population genetic structure. Regarding introduction scenarios, we hypothesised that Portuguese *A. longifolia* populations originated from a single introduction event from Australia, whereas in South Africa, we expected our modelling results to support the known multiple and independent introductions of the species into the country (Poynton 2009). We thus hypothesised that this invasion would be characterised by high levels of genetic admixture. We had no clear prediction for the introduction history of *A. longifolia* in South America given the scarcity of historical information for the region.

Methods

Collection of Plant Material and DNA Extraction

Phyllodes of *Acacia longifolia* were collected from individual plants in several invasive [Portugal, POR; Spain – Galicia, ESP; South Africa, RSA; Brazil, BRA (Vicente et al. 2020); and Uruguay, URU] and native (mainland southeast Australia, AUS; and Tasmania, TAS) range populations. Within each country we sampled more than one popula-

tion where possible (see Table 1). In Portugal, individuals from Vila Nova de Milfontes (VNMF), Pinheiro da Cruz (PC) and two individuals from Osso da Baleia (included in the Mira population) were previously sampled by Vicente et al. (2018). In Australia, additional material was obtained from seedlings grown from seeds acquired from local nurseries. In total, 272 individuals were included in our analyses (Table 1, Fig. 1A), 126 from invaded ranges (Table 1A, Fig. 1B–D) and 146 from Australia (Table 1B, Fig. 2).

DNA was extracted using the method of Doyle and Doyle (1987), as modified by Weising et al. (1994) and adapted for *A. longifolia* by Vicente et al. (2018). DNA was quantified using spectrophotometry.

Microsatellite amplification and genotyping

Ten chloroplast microsatellite loci (cpSSRs; ccmp1-10) described by Weising and Gardner (1999) were tested for cross-amplification in *A. longifolia* (Suppl. material 1: table S1). For these cross-amplification tests, we first used the PCR conditions described by Weising and Gardner (1999) and, when needed, adjusted $MgCl_2$ concentrations between 1.5 mM to 2.5 mM and annealing temperatures between 50 °C to 52 °C to optimise amplifications of individual loci. Loci were selected based on visualisation of PCR products on 2% agarose gels. Four loci (ccmp3, 4, 5 and 7) consistently yielded high quality amplicons and were selected for amplification in all samples. Details of PCR primers can be found in Suppl. material 1: table S2. For cpSSRs each 15 µL reaction contained 20ng DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix (Promega, USA), 0.5 µM fluorescently labelled (with either 6-FAM or HEX) forward primer and 0.5 µM reverse primer (STAB Vida, Portugal), 1 U of GoTaq Flexi DNA polymerase and 1× colourless GoTaq Flexi buffer (Promega, USA). The following thermal cycle was used: initial denaturation at 94 °C for 5 min, followed by 40 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, with a final extension period of 8 min at 72 °C. As a routine procedure, each reaction included a negative control that did not include DNA and at least 10% of repeated samples that were run in a 2% agarose gel to confirm amplification before fragment analysis.

We searched the literature for studies that have developed nuclear microsatellites (nSSRs) in *Acacia* species. We also tested primers that we designed for *A. cyclops* (unpublished data). This led to a total of 32 primer pairs that were screened for cross-amplification in *A. longifolia* (Suppl. material 1: table S1). For optimisation, $MgCl_2$ concentrations were varied between 1.5 mM and 2.5 mM, annealing temperature between 52 °C and 60 °C, extension times between 15s and 30s, and primer concentrations between 0.1 µM and 0.2 µM. Loci were selected based on visualisation of PCR amplification products on 2% agarose gels. Eleven nSSR loci were retained for amplification in our samples. Primers for locus DCLOC (Roberts et al. 2013) were previously optimised for analysis in *A. longifolia* by Vicente et al. (2018) and were also included in this study. Genotyping data for this locus for samples from Vila Nova de Milfontes (VNMF), Pinheiro da Cruz (PC) and two samples from Osso da Baleia (included in the Mira population) were obtained from Vicente et al. (2018) and incorporated into

Table 1. Number of samples (n), population code, latitude, and longitude of the collection sites. **A** invasive range (Brazil, Portugal, South Africa, Spain, and Uruguay) **B** native range (mainland Australia and Tasmania). Collection sites in mainland Australia in **bold** represent seedling samples obtained from nurseries.

		1A		
Collection Site	**Code**	**N**	**Latitude / Longitude**	**Sampling year**
Portugal	***POR***	***38***		
Vila Nova de Milfontes	VNMF	5	37.685292, -8.791508	2015
Vila Nova de Milfontes			37.675608, -8.766397	2015
Vila Nova de Milfontes			37.511822, -8.440267	2015
Pinheiro da Cruz	PC	6	38.250377, -8.752181	2015
Osso da Baleia	Mira	7	40.000884, -8.901132	2015
Mira			40.527170, -8.673730	2018
Mira			40.450170, -8.768960	2018
Mira			40.461380, -8.708500	2018
Foz do Arelho	FA	6	39.429354, -9.223632	2019
Monte Gordo	MG	6	37.183880, -7.448606	2019
Moledo	Mol	8	41.866359, -8.855380	2017
Spain (Galicia)	***ESP***	***12***		
Muros	Muros	6	42.820272, -9.065278	2019
San Vicente	SanVic	6	42.464995, -8.908974	2019
South Africa	***RSA***	***36***		
Stellenbosch	Stell	5	-33.947222, 18.834920	2017
Grahamstown	Graham	6	-33.327920, 26.499520	2018
Grahamstown			-33.321640, -33.32164	2018
Clarkson	Clark	6	-34.071800, 24.404570	2018
R102			-33.981450, 24.043820	2018
Sedgefield	Sedge	8	-34.068380, 22.948030	2018
Hermanus	Herm	5	-34.395970, 19.218410	2018
Lasikisiki	Lasiki	6	-31.413750, 29.712980	2018
Brazil	***BRA***	***25***		
Tramandaí	Tram	5	-29.890618, -50.097749	2019
Cassino Beach	Cass	5	-32.188509, -52.169312	2019
Hermenegildo	Hmng	5	-33.639901, -53.420143	2019
Lagoa do Peixe National Park	Peixe	5	-31.250075, -51.026285	2019
Moçambique Beach	Moca	5	-27.486697, -48.393998	2019
Uruguay	***URU***	***15***		
Cabo Polonio	Polonio	5	-34.407141, -53.878037	2019
Hotel Paque Oceánico Beach	Hotel	5	-33.908477, -53.512534	2019
Brazil/Uruguay Frontier	Front	5	-33.728768, -53.468794	2019
		1B		
Mainland Australia	***AUS***	***76***		
Clovelly, NSW	Clov	8	-33.914732, 151.263171	2017
Green Point, NSW	Green	8	-32.250278, 152.536667	2020
Bilpin, NSW	Bilpin	6	-33.491667, 150.533333	2020
Ulladulla, NSW	Ulladulla	8	-35.350000, 150.483333	2020
Vaucluse, NSW	Vaucluse	8	-33.852778, 151.263889	2020
Torrington, NSW	Torrington	6	-29.207500, 151.686389	2020
Marulan, NSW	Marulan	8	-34.683333, 150.066667	2020
Beachport, SA	Beachport	8	-37.516667, 140.083333	2020
Curdievale, VIC	Curdievale	7	-38.508123, 142.899504	2020
Bermagui, NSW	Bermagui	9	-36.443373, 150.061346	2020

1B				
Collection Site	**Code**	**N**	**Latitude / Longitude**	**Sampling year**
Tasmania	***TAS***	***70***		
Bridport	Bridport	8	-40.999805, 147.393570	2020
St. Helens Conservation Area (Private property)	Helens	8	-41.328235, 148.294909	2020
Seven Mile Beach	SMile	8	-42.850198, 147.522249	2020
Southwest National Park	SouthW	8	-43.606146, 146.817540	2020
Three Sisters National Park	ThreeS	8	-41.129030, 146.125828	2020
Freycinet National Park	Freycinet	8	-42.173890, 148.279790	2020
Whale Bone Point	Whale	8	-43.439267, 147.235150	2020
Stanley	Stanley	8	-40.780950, 145.277317	2020
Arthur River	Arthur	6	-41.033333, 144.666667	2020

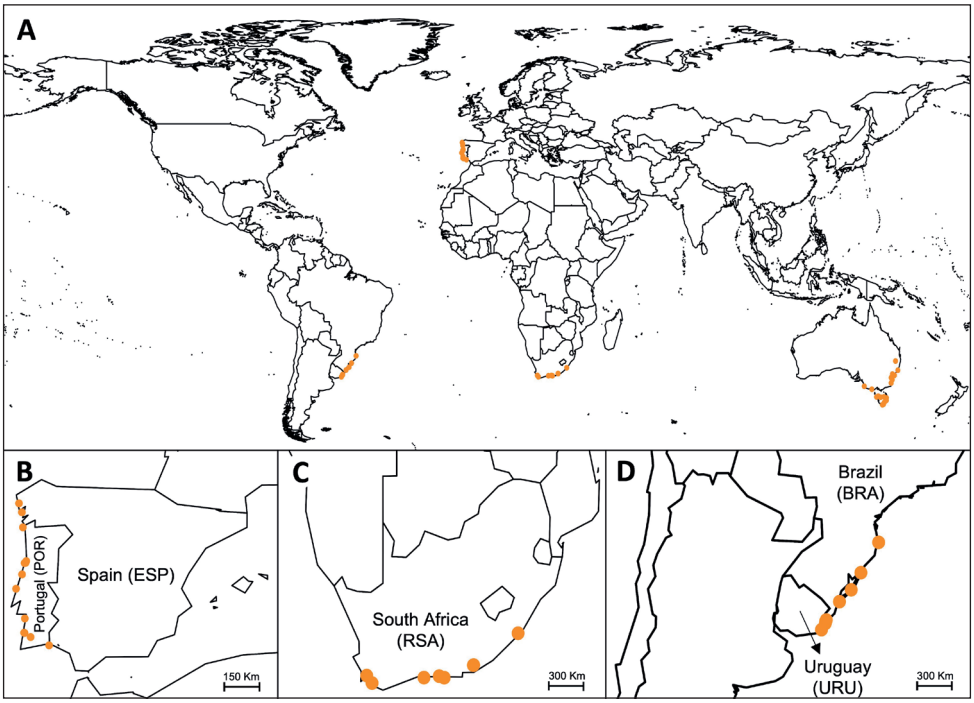

Figure 1. Map of the collection sites of *Acacia longifolia*. Each dot represents the exact location of each collection site (see coordinates in Table 1) **A** all sampled countries/locations **B** Iberian Peninsula **C** South Africa **D** Brazil and Uruguay. Map was drawn using the *maptools* R package (Lewin-Koh et al. 2011; R Core Team 2016).

the dataset generated in this study. Thus, in total, 12 nSSR loci were analysed in this study (see Suppl. material 1: table S2 for further details).

PCRs of nSSR loci were performed in 15 μL reaction volumes, each containing 10 ng DNA, 1.5–2.5 mM of $MgCl_2$ depending on primers used (Suppl. material 1: table S2), 0.2 mM dNTP mix (Promega, USA), 0.2 μM primer forward labelled with

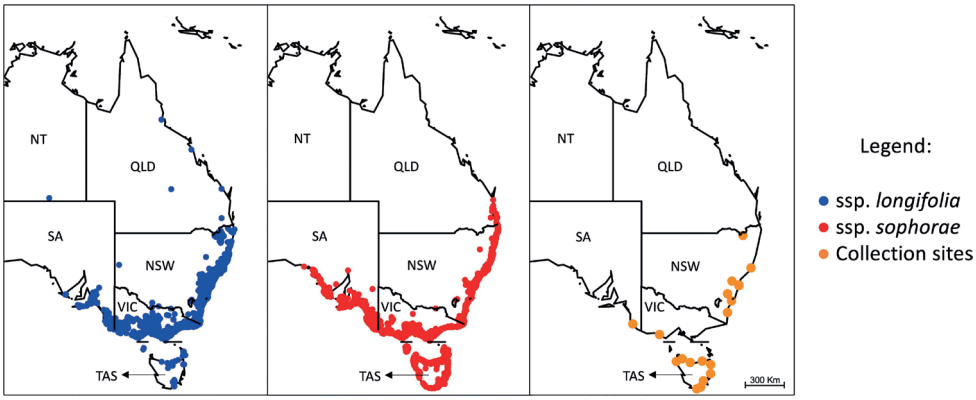

Figure 2. Maps of the native range distribution of *Acacia longifolia*. Distributions of *A. l.* ssp. *longifolia* and *A. l.* ssp. *sophorae* are presented in blue and red, respectively, and the sample collection sites of this study are presented in orange. Each orange dot represents the exact location of each collection site (see Table 1). Maps were drawn using the *maptools* R package (Lewin-Koh et al. 2011; R Core Team 2016). Geo-referenced occurrence records for each subspecies were obtained from the Atlas of Living Australia database (ALA 2021a, b) and the Global Biodiversity Information Facility (GBIF 2021a,b), with duplicates and erroneous records (i.e., records with coordinates that fell in the ocean or with no registered coordinates) manually removed in R statistical environment (R Core Team 2016) with the *maptools*, *raster* (Hijmans and van Etten 2010) and *rgdal* (Keitt et al. 2010) R packages. NSW: New South Wales. NT: Northern Territory. QLD: Queensland. SA: South Australia. TAS: Tasmania. VIC: Victoria.

a fluorescent dye (either 6-FAM, HEX or ATTO-550), 0.2 μM reverse primer (STAB Vida, Portugal), 1 U of GoTaq Flexi DNA polymerase and 1× colourless GoTaq Flexi buffer (Promega, USA). The PCR cycle consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 30 sec at 94 °C, 1 min at the annealing temperature optimised for each primer pair (Suppl. material 1: table S2), and 30 sec at 72 °C, with a final extension period of 7 min at 72 °C. For the two primer pairs, Division/Bell and The/Wall, extension time was reduced to 15 s. Positive and negative controls were included in each reaction, as explained above. Fragments for all loci were separated on an Applied Biosystems 3100 Series System with the Dye Set DS-30 (internal standard ROX) by STAB Vida (Portugal) and scored using Peak Scanner in the ThermoFisher AppConnect online software portal (ThermoFisher Scientific, USA).

Population genetic diversity and structure

Haplotypes were identified based on the combination of alleles across all cpSSR loci/individual (see Results section). The number of different alleles (N_a), number of effective alleles (N_e), Shannon's Information Index (I), and haplotype diversity (h) were calculated using GenAlEx v6.5 (Peakall and Smouse 2006, 2012). The Network v10.2 software (www.fluxus-engineering.com) was used to draw a median-joining (MJ; Bandelt et al. 1999) haplotype network.

Micro-Checker v2.2 (Van Oosterhout et al. 2004) was used to check for scoring errors and large allele dropouts in our nSSR genotype dataset. This software was also used to generate a dataset that has been corrected for null alleles, using the Oosterhout algorithm with 95% confidence intervals (Van Oosterhout et al. 2004). Expected heterozygosity (H_E), observed heterozygosity (H_O), allelic richness (A_r), and inbreeding coefficients (F_{IS}) were calculated for the corrected and uncorrected genotype datasets using the *diveRsity* R package (Keenan et al. 2013). These metrics were compared between datasets using the independent 2-group Mann-Whitney U test in R environment (R Core Team 2016) to check if the presence of null alleles affected our results. The mean frequency of null alleles across all loci and populations was calculated with the FreeNA software (Chapuis and Estoup 2007), as well as the ENA-corrected (i.e., excluding null alleles; ENA method, Chapuis and Estoup 2007) and uncorrected global (i.e., at each locus and population) and pairwise population fixation indices (F_{ST}). To further check for the effects of null alleles on our analyses, statistical differences between the ENA-corrected and uncorrected global and pairwise F_{ST} values were tested using the independent 2-group Mann-Whitney U test in R environment (R Core Team 2016). Since null alleles had an overall low mean frequency and did not significantly affect any of the calculated metrics (see Results), no correction for null alleles was applied and all further analyses were performed using the uncorrected nSSR genotype dataset. Allele frequency departures from Hardy-Weinberg equilibrium (HWE) for all loci were tested using GenAlEx v6.5 (Peakall and Smouse 2006, 2012).

The four descriptive population diversity metrics mentioned above (H_E, H_O, A_r, and F_{IS}) were compared among countries using a Kruskal-Wallis rank sum test in R environment (R Core Team 2016). In this analysis, individuals from Spain were excluded as only two populations were sampled in this country. Instead, the analysis was repeated by grouping the samples from Spain with the ones from Portugal (i.e., Iberian Peninsula; IBP).

Bayesian assignment tests were performed using the STRUCTURE v2.3.4 program (Pritchard et al. 2000) to investigate the genetic structure of the native and invasive *A. longifolia* populations. An admixture model with correlated allele frequencies was used to test for the number of genetic clusters (K), ranging from 1 to 15, with 100,000 burn-in iterations, 250,000 Markov Chain Monte Carlo repetitions (MCMC), and 15 iterations per K value. We then performed a hierarchical analysis by removing samples belonging to the smallest genetic cluster identified by this analysis (13 samples belonging to Portugal and South Africa; see Results) to infer structure among the remaining populations. We repeated this analysis with the same conditions as above, but with K ranging from 1 to 10. For all STRUCTURE analyses the optimum number of genetic clusters was evaluated in STRUCTURE HARVESTER web v0.6.94 (Earl and vonHoldt 2012) by applying the delta K method of Evanno et al. (2005) and also analysing the likelihood distributions [LnP(K)]. Graphical visualisations of these results were obtained using the CLUMPAK server main pipeline (Kopelman et al. 2015).

To better understand population structure within the native range, we also ran a separate STRUCTURE analysis that included data from native range individuals only, with *K* ranging from 1 to 10, implementing an admixture model with correlated allele frequencies using 10,000 burn-in iterations, 50,000 MCMC repetitions, and 10 iterations per *K* value. The optimum number of genetic clusters and the graphical visualisation of the results were obtained as described above. This analysis identified $K = 2$ as the optimum number of genetic clusters, with one cluster predominantly corresponding to mainland Australia and the other predominantly to Tasmanian populations (see Results). To check for population genetic substructure within these two identified clusters, ancestry coefficients for each individual were averaged by cluster over the 10 iterations for $K = 2$, and individuals having a minimum ancestry coefficient cut-off value of 0.7 for their corresponding cluster were selected for further cluster-specific (i.e., mainland Australia or Tasmania) analyses. We used the same parameters described above to identify the optimum number of genetic clusters and to graphically visualise these. We also tested for Isolation by Distance (IBD) by performing a Mantel test with 9,999 permutations using the *ade4* R package (Dray et al. 2022) by comparing the linearised pairwise F_{ST} values matrix (i.e., $F_{ST}/1\text{-}F_{ST}$) with the geographical distance matrix obtained with the Geographic Distance Matrix Generator v1.2.3 software (Ersts 2022). We compared the mean pairwise F_{ST} values among populations within mainland Australia, populations within Tasmania, and populations from mainland Australia *vs* Tasmania using a Kruskal-Wallis rank sum test followed by a post-hoc independent 2-group Mann-Whitney U test in R environment (R Core Team 2016).

Inference of invasion sources

Approximate Bayesian Computations (ABC) were performed in the DIYABC v2.1.0 software (Cornuet et al. 2014) to test different invasion scenarios for each invaded country separately. In these analyses, we took the two major genetic clusters in the native range identified by the STRUCTURE analysis (see Results) into consideration, i.e., populations from mainland Australia (AUS) and Tasmania (TAS), while invaded populations that were pooled by country. All tested scenarios assumed that the AUS and TAS populations diverged from a native ancestral unsampled population, with the following variations: 1) the invasive population originated from the AUS populations; 2) the invasive population originated from the TAS populations; 3) the invasive population originated from an admixed unsampled population resulting from multiple introductions which originated from both AUS and TAS populations; 4) the invasive population originated from multiple introductions ("ghost" unsampled populations) originating from AUS populations; 5) the invasive population originated from multiple introductions ("ghost" unsampled populations) originating from TAS populations; and 6) the invasive population originated from an unknown native population which is related to the AUS and TAS genetic lineages (see Fig. 3). We ran 3×10^6 simulations for each scenario for each invaded country. We did not consider any genetic bottlenecks in these scenarios, as we found no evidence for their existence (see Results section).

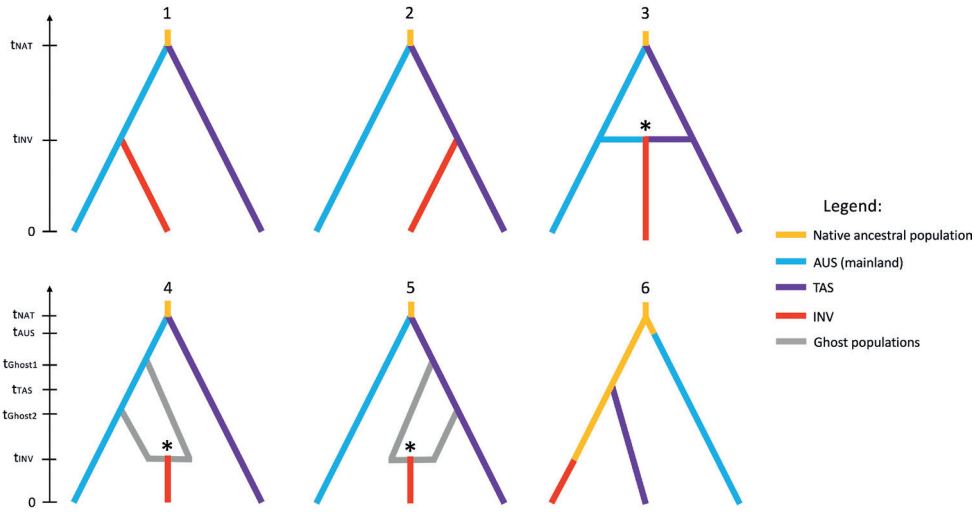

Figure 3. Diagrams of the six tested scenarios for each invaded country via DIYABC analyses. Asterisks represent merging and admixture of two populations. The "native ancestral population" is an unsampled population genetically related to both the mainland south-eastern Australia (AUS) and Tasmania (TAS) clusters identified via STRUCTURE analyses. "Ghost populations": unsampled populations. INV: Invasive population. See Materials and Methods for detailed descriptions of each scenario. Parameter descriptions, prior and posterior values are provided in Suppl. material 1: tables S3–S5.

The summary statistics selected for the models were the following: mean number of alleles, mean genetic diversity, mean allele size variance, mean M index, fixation index (F_{ST}), mean index of classification, shared allele distance and $\delta\mu^2$ genetic distance. The description of the parameters, their prior distributions, and parameter rules are provided in Suppl. material 1: tables S3–S5. The priors for time of invasion (t_{INV}) varied between countries according to the latest year of sample collection and the year of first introduction reported in historical records and literature (see Introduction for details), assuming a generation time of 2–3 years (Maslin and McDonald 2004; Vicente et al. 2021). For the purposes of this analysis, since there is no available information on a specific introduction year into Spain (Galicia), the year of introduction into Portugal was assumed to coincide with the introduction of *A. longifolia* into Spain (see Introduction for details). This allowed an estimation of the maximum possible number of generations that *A. longifolia* has been present in each invaded country. The posterior probabilities of each scenario were calculated using a logistic approach on the 1% simulated datasets that were closest to the observed dataset. For the scenario(s) with the highest probability for each country, the posterior distribution of parameters was estimated using a logit transformation on the 1% simulated datasets that were closest to the observed dataset. The precision of these parameters was assessed using the "bias and precision" option on 500 test datasets to estimate the relative median absolute deviation (RMedAD) and means of the median relative bias (MedRB). The adequacy of the chosen scenario(s) was assessed using the "model checking" option by simulat-

ing 1000 datasets using a logit transformation of parameters, with a different set of summary statistics to avoid overestimating the fit of the model. The chosen statistics were the mean number of alleles, mean genetic diversity, mean allele size variance, and $\delta\mu^2$ genetic distance. Type I and Type II errors were also computed for the scenarios(s) with the highest posterior probability using the "confidence in scenario choice" option using a logistic regression and simulating 100 test datasets.

Due to the identification of a cluster connecting South Africa and Portugal (see Results section), we also tested 6 scenarios with data from these two countries, that correspond to scenarios 1, 2 and 6 described above with and without a bridgehead between South Africa and Portugal. We ran simulations and "model checking" analyses as described above for non-bridgehead scenarios.

Results

Dataset characteristics

We identified between two and three alleles per cpSSR locus (mean 2.5), ranging between 93–154 bp in size. We identified between two and nine alleles per nSSR locus (mean 5.08), ranging between 81 bp to 328 bp in size. Scoring errors due to stuttering were found for locus C51M0 (Mira population, POR; Table 1A) and locus AH3-1 (Seven Mile beach population, TAS; Table 1B). All loci showed departures from Hardy-Weinberg equilibrium, and within populations some were monomorphic. However, mean null allele frequency was low in our dataset (mean = 0.027; SD = 0.073) and ENA-corrected and uncorrected F_{ST} values were not significantly different between null allele-corrected and uncorrected datasets for both global and pairwise estimates (Mann-Whitney U test $p > 0.5$; Suppl. material 1: fig. S1). Similarly, population diversity metrics (H_E, H_O, A_r, and F_{IS}) were not significantly different between the corrected and uncorrected datasets (Mann-Whitney U test $p > 0.5$; Suppl. material 1: fig. S2). Therefore, null allele corrections were not considered in all further nSSR analyses.

Population genetic diversity and structure

Our cpSSR data identified eight unique haplotypes (hereafter haplotypes A–H; Fig. 4A). Haplotype E was the most dominant in invasive ranges, while haplotype D was the most frequent one in the native range. Haplotypes B, C and G only occurred in the native range. Portugal and South Africa shared one haplotype (A), and also had a unique haplotype each (F and H, respectively). Our network analysis found that haplotypes B, D and E were closely related to most other haplotypes (Fig. 4B). The number of different alleles (N_a) ranged from 1 (BRA and ESP) to 2.25 (RSA; Table 2), while the number of effective alleles (N_e) ranged from 1 to 1.659 (similar to *Na*; Table 2). In invaded countries, South African populations had the highest haplotype diversity (h = 0.407, Table 2), while Spanish and Brazilian populations had a single

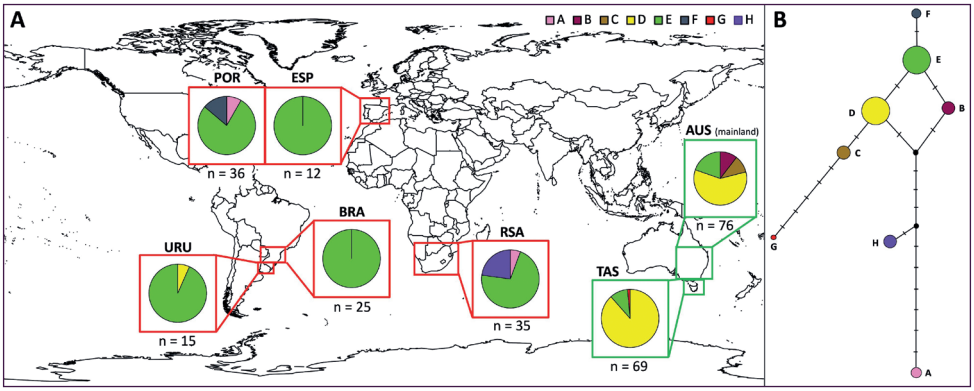

Figure 4. Haplotypes based on chloroplast microsatellites **A** geographical distribution of the eight identified haplotypes (A–H). The number of samples (n) is shown below pie charts **B** median-joining network analysis of eight chloroplast haplotypes. The size of each circle is proportional to the frequency of each haplotype, and branch markings indicate mutational steps between haplotypes.

Table 2. Diversity metrics of all native and invasive *Acacia longifolia* populations for chloroplast microsatellites. N_a – Number of different alleles; N_e – Number of effective alleles; h – Haplotype diversity. Standard error (SE) is shown in parenthesis.

Range	Country	N_a	N_e	H
Invasive	POR	2.000 (0.408)	1.229 (0.119)	0.165 (0.074)
Invasive	ESP	1.000 (0.000)	1.000 (0.000)	0.000 (0.000)
Invasive	RSA	2.250 (0.250)	1.659 (0.049)	0.396 (0.018)
Invasive	BRA	1.000 (0.000)	1.000 (0.000)	0.000 (0.000)
Invasive	URU	1.250 (0.250)	1.036 (0.036)	0.031 (0.031)
Native	AUS	1.750 (0.479)	1.360 (0.287)	0.184 (0.129)
Native	TAS	1.750 (0.479)	1.073 (0.054)	0.059 (0.050)

haplotype (Table 2). Haplotype diversity in Australian populations ranged between 0.06 (TAS) to 0.186 (AUS; Table 2). Haplotype B was only found in South Australia (SA; Beachport population, Table 1B) while haplotype G occurred only in Tasmania (TAS; St. Helens Conservation Area population, Table 1B). Haplotype E was found in New South Wales (NSW; Clovelly and Torrington populations, Table 1B) and in one population in eastern Tasmania (St. Helens Conservation Area population). Haplotype C was predominantly restricted to mainland Australia, while haplotype D was widespread across the native range.

For invasive populations, and for nSSRs, mean expected heterozygosity (H_E) ranged from 0.29 (ESP) to 0.33 (POR), while mean observed heterozygosity (H_O) ranged from 0.33 (RSA) to 0.40 (BRA), mean allelic richness (A_r) ranged from 1.74 (ESP) to 1.97 (POR), and mean inbreeding coefficient (F_{IS}) ranged from -0.146 (RSA) to -0.317 (BRA; Table 3). In the native range, mean H_E ranged from 0.25 (AUS) to 0.27 (TAS), while mean H_O ranged from 0.31 (TAS) to 0.32 (AUS), mean A_r ranged from 1.73 (AUS) to 1.77 (TAS), and mean F_{IS} ranged from -0.196 (TAS) to -0.303

Table 3. Diversity metrics of all native and invasive populations of *Acacia longifolia* for nuclear microsatellites. H_E – Expected heterozygosity; H_O – Observed heterozygosity; A_r – Allelic richness; F_{IS} – Inbreeding coefficient; SD – Standard deviation.

Range	Country	Population	H_E	H_O	A_r	F_{IS}
Invasive	POR	VNMF	0.29	0.35	1.84	-0.222
Invasive	POR	PC	0.29	0.42	1.75	-0.458
Invasive	POR	Mira	0.53	0.32	2.70	0.397
Invasive	POR	FA	0.28	0.36	1.75	-0.268
Invasive	POR	MG	0.26	0.33	1.75	-0.292
Invasive	POR	Mol	0.31	0.39	2.01	-0.247
		Mean	***0.33***	***0.36***	***1.97***	***-0.181***
		SD	***0.10***	***0.04***	***0.37***	***0.294***
Invasive	ESP	Muros	0.31	0.37	1.76	-0.176
Invasive	ESP	SanVic	0.27	0.32	1.72	-0.195
		Mean	***0.29***	***0.35***	***1.74***	***-0.185***
		SD	***0.03***	***0.04***	***0.03***	***0.014***
Invasive	RSA	Stell	0.24	0.37	1.59	-0.529
Invasive	RSA	Graham	0.31	0.38	1.86	-0.209
Invasive	RSA	Clark	0.45	0.40	2.37	0.112
Invasive	RSA	Sedge	0.22	0.31	1.51	-0.436
Invasive	RSA	Herm	0.23	0.20	1.55	0.126
Invasive	RSA	Lasiki	0.32	0.31	1.91	0.059
		Mean	***0.30***	***0.33***	***1.80***	***-0.146***
		SD	***0.09***	***0.07***	***0.33***	***0.288***
Invasive	BRA	Tram	0.33	0.35	1.95	-0.050
Invasive	BRA	Cass	0.32	0.42	2.01	-0.289
Invasive	BRA	Hmng	0.32	0.47	2.03	-0.443
Invasive	BRA	Peixe	0.31	0.40	1.83	-0.283
Invasive	BRA	Moca	0.24	0.37	1.60	-0.517
		Mean	***0.30***	***0.40***	***1.88***	***-0.317***
		SD	***0.04***	***0.05***	***0.18***	***0.180***
Invasive	URU	Polonio	0.33	0.38	1.98	-0.162
Invasive	URU	Hotel	0.29	0.35	1.73	-0.221
Invasive	URU	Front	0.28	0.38	1.76	-0.386
		Mean	***0.30***	***0.37***	***1.82***	***-0.256***
		SD	***0.03***	***0.02***	***0.14***	***0.116***
Native	AUS	Clov	0.31	0.38	1.85	-0.216
Native	AUS	Green	0.26	0.31	1.73	-0.179
Native	AUS	Bilpin	0.27	0.31	1.70	-0.133
Native	AUS	Ulladulla	0.21	0.33	1.60	-0.558
Native	AUS	Vaucluse	0.27	0.39	1.79	-0.441
Native	AUS	Torrington	0.12	0.18	1.29	-0.472
Native	AUS	Marulan	0.34	0.43	2.18	-0.247
Native	AUS	Beachport	0.21	0.25	1.57	-0.189
Native	AUS	Curdievale	0.30	0.37	1.82	-0.226
Native	AUS	Bermagui	0.26	0.33	1.88	-0.283
		Mean	***0.25***	***0.32***	***1.73***	***-0.303***
		SD	***0.06***	***0.07***	***0.23***	***0.144***
Native	TAS	Bridport	0.20	0.26	1.56	-0.342
Native	TAS	Helens	0.35	0.43	2.05	-0.256
Native	TAS	SMile	0.38	0.40	2.23	-0.068

Range	Country	Population	H_E	H_O	A_r	F_{IS}
Native	TAS	SouthW	0.27	0.36	1.76	-0.346
Native	TAS	ThreeS	0.28	0.34	1.80	-0.218
Native	TAS	Freycinet	0.25	0.20	1.70	0.196
Native	TAS	Whale	0.26	0.30	1.68	-0.190
Native	TAS	Stanley	0.19	0.18	1.57	0.086
Native	TAS	Arthur	0.22	0.36	1.62	-0.625
		Mean	***0.27***	***0.31***	***1.77***	***-0.196***
		SD	***0.06***	***0.09***	***0.23***	***0.245***

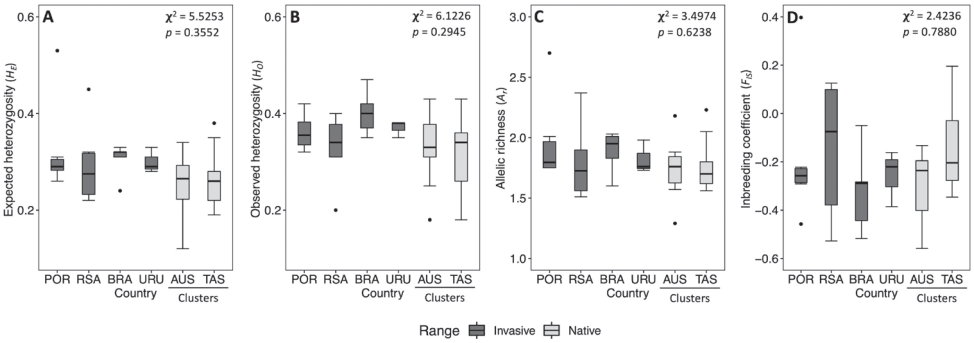

Figure 5. Comparisons of diversity metrics of *Acacia longifolia* between invaded countries and two native range genetic clusters **A** expected heterozygosity **B** observed heterozygosity **C** allelic richness **D** inbreeding coefficient. The two native range clusters were identified via STRUCTURE analysis. Kruskal-Wallis test results are shown on the upper right corner, with 5 degrees of freedom. Spain was excluded from the analysis due to the low number of populations sampled.

(AUS; Table 3). Diversity metric comparisons (excluding Spanish populations due to low population numbers) found similar levels of genetic diversity and lack of inbreeding between native and different invasive ranges (Kruskal-Wallis test $p > 0.05$ for all diversity metrics; Fig. 5). Similar results were obtained when these analyses were repeated with individuals from Spain and Portugal lumped together as Iberian Peninsula (IBP; Suppl. material 1: fig. S3).

Our initial STRUCTURE analysis based on all nSSR data identified two genetic clusters (Suppl. material 1: fig. S4A, B), one that included individuals from Portugal (Mira population) and South Africa (Clarkson and Sedgefield populations) and another that included all other populations (Fig. 6A). STRUCTURE analysis of the latter cluster (i.e., hierarchical analysis) revealed two further genetic subclusters (Suppl. material 1: fig. S4C, D). In this second analysis, native range populations separated in two genetic clusters roughly corresponding to those from mainland Australian (AUS) and Tasmania (TAS), while populations from the invaded ranges were admixtures of these two genetic clusters (Fig. 6B, and see Suppl. material 1: fig. S5 for bar plots of $K = 3 – 6$).

Analysis of the native range-only data also identified two genetic clusters (Suppl. material 1: fig. S6), corresponding to mainland Australia and Tasmania (Fig. 7A). We

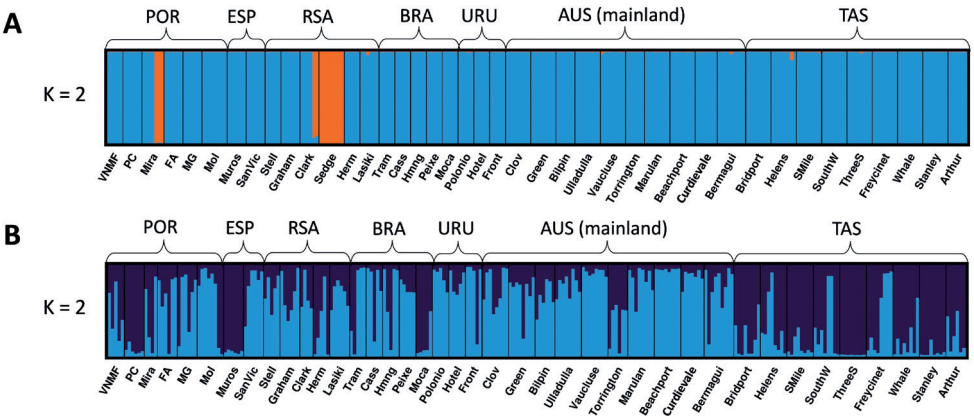

Figure 6. STRUCTURE bar plots ($K = 2$) in the invasive and native ranges of *Acacia longifolia* **A** bar plot for the complete dataset **B** bar plot for the hierarchical analysis of the blue cluster in A. Population names underneath the plots correspond to the codes provided in Table 1.

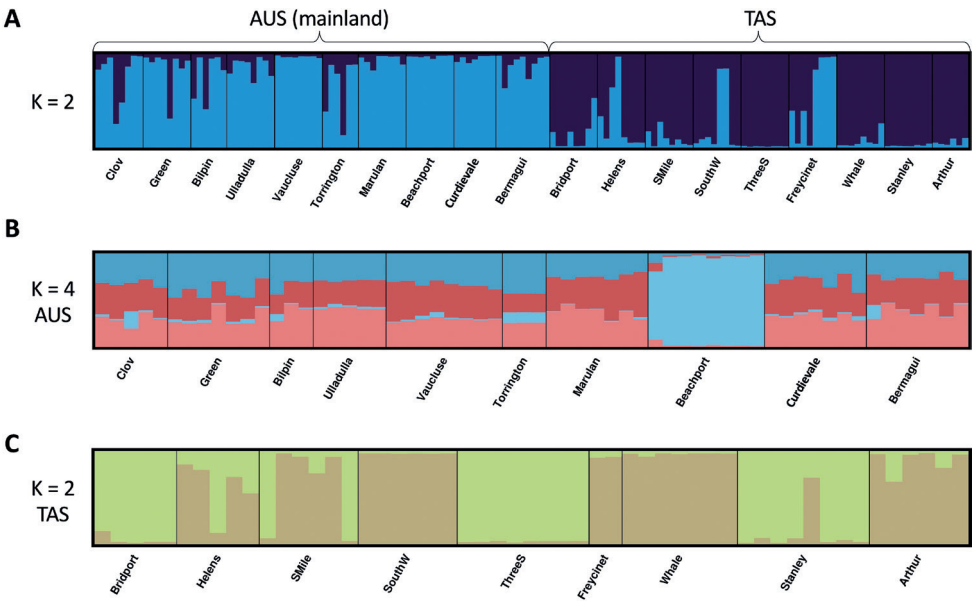

Figure 7. STRUCTURE bar plots for the identified optimum number of clusters in *Acacia longifolia*'s native range **A** bar plot for the overall native range ($K = 2$) **B** bar plot for the mainland Australia cluster ($K = 4$) **C** bar plot for the Tasmania cluster ($K = 2$). Population names underneath the plots correspond to the codes provided in Table 1.

identified significant IBD (Mantel test r = 0.159, $p = 0.04$) and found pairwise fixation indices among populations within mainland Australia and within Tasmania to be significantly lower than the fixation indices among populations from mainland Australia and Tasmania (Fig. 8, Kruskal-Wallis test $\chi^2 = 12.848$, $p = 0.002$, followed by a

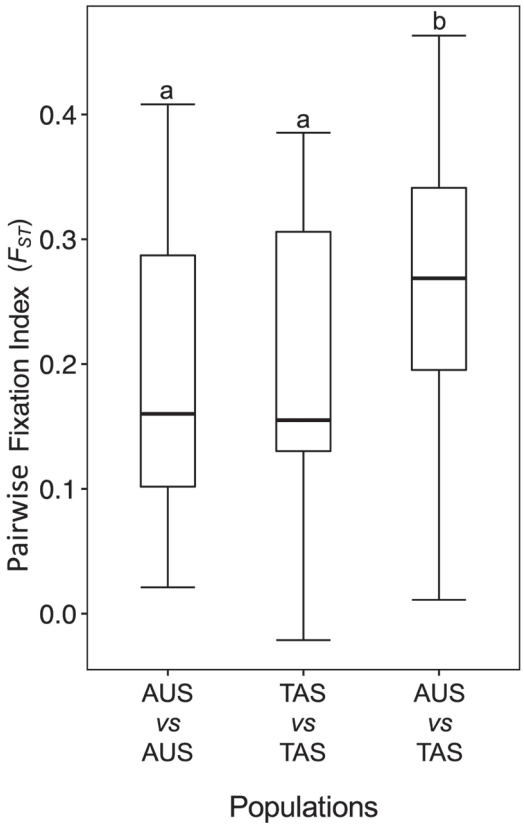

Figure 8. Comparison of pairwise fixation indices (F_{ST}) among Australian *Acacia longifolia* populations. Comparisons were made among populations within mainland Australia (AUS vs AUS), populations within Tasmania (TAS vs TAS), and among population of mainland Australia vs Tasmania (AUS vs TAS). Kruskal-Wallis test $p < 0.05$, and letters show the result of the post-hoc Mann-Whitney U test.

post-hoc Mann-Whitney U test), further supporting the STRUCTURE assignment of individuals to two genetic clusters corresponding to mainland Australia and Tasmania. However, higher values of *K* suggested that substructure existed within these two clusters (Suppl. material 1: fig. S7). Hierarchical STRUCTURE analyses revealed four clusters within mainland Australia and two clusters within Tasmania (Fig. 7B, C, respectively; Suppl. material 1: fig. S8A, B and C, D, respectively). Within mainland Australia, the population from Beachport appears to be distinct from all other populations (Fig. 7B; it also has a unique cpSSR haplotype B, Fig. 4), while all other populations show equal and symmetrical levels of admixture (i.e., being panmictic; also visible for other higher *K* values, Suppl. material 1: fig. S9). Within Tasmania, the Bridport, Three Sisters National Park, and Stanley populations seem to be genetically distinct from all other populations (Fig. 7C), with the Three Sisters National Park population being the most distinct (Suppl. material 1: fig. S10, $K = 6$). Bar plots for higher values of *K* are presented in the Suppl. material 1 (Suppl. material 1: figs S9, S10).

Inference of invasion sources

A similar introduction scenario was inferred for South Africa and Portugal, with the origin of invasive populations being an unknown population related to both the AUS and TAS genetic lineages (i.e., scenario 6, Fig. 3). This scenario had very high probability for both countries (p = 0.997, 95% CI = 0.995–0.998, and p = 0.928, 95% CI = 0.906–0.95, respectively; Suppl. material 1: fig. S11). Similar introduction scenarios were identified for Brazil and Uruguay: single or multiple introductions from Tasmania, i.e., scenarios 2 and 5, respectively (Fig. 3). Statistical support for these scenarios was lower for Brazil (scenario 2: p = 0.417, 95% CI = 0.391–0.443; scenario 5: p = 0.425, 95% CI = 0.399–0.451; Suppl. material 1: fig. S11) and Uruguay (scenario 2: p = 0.403, 95% CI = 0.378–0.429; scenario 5: p = 0.418, 95% CI = 0.392–0.444; Suppl. material 1: fig. S11) compared with the support for the most likely scenarios for Portugal and South Africa. Our ABC analysis for Spain was inconclusive, thus any discussion (see below) regarding these populations does not rely on this result.

Based on the scenarios with highest posterior probability for each invaded country, several parameters such as effective population size, time of events (e.g., introductions), admixture, and mutation rates were computed (Suppl. material 1: table S3). The mean time since first introduction of *A. longifolia* into South Africa and Portugal was 53.4 and 34.1 generations, respectively (Suppl. material 1: table S4). The mean time since first introduction ranged from 18.6 to 29.6 generations for Uruguay and from 13.8 to 16.3 generations for Brazil, depending on the scenario (2 or 5; Suppl. material 1: table S5). Bias values (i.e., RMedAD and MedRB) indicated that our model estimates are plausible (Suppl. material 1: tables S4, S5). Low Type I and Type II errors were inferred for both the Portuguese and South African scenarios (ranging from 0.02 to 0.12; Suppl. material 1: table S6). Conversely, the scenarios for Brazil and Uruguay showed higher Type I and Type II errors (ranging from 0.45 to 0.60; Suppl. material 1: table S6), but their posterior probabilities were low (compared with the scenario with highest probability for Portugal and South Africa). Results for the adequacy of the scenarios (i.e., "model checking", Suppl. material 1: table S7) showed that all high posterior probability scenarios had a slight deviation between the observed and simulated mean allelic size variance statistic, and in some cases also the mean genetic distance, but overall indicate that these scenarios sufficiently explained the observed data.

Results from the bridgehead scenario analysis (Suppl. material 1: fig. S12) showed that scenario 5 had the highest posterior probability (p = 0.716, 95% CI = 0.617–0.818). This scenario (Suppl. material 1: fig. S13) represents independent introductions of *A. longifolia* into South Africa and Portugal from an unknown native source that is related to both the AUS and TAS genetic lineages, thus not supporting a bridgehead introduction event between these two countries. The "model checking" analysis (Suppl. material 1: table S8) showed many deviations between the observed and simulated values of several summary statistics, indicating that this scenario does not explain our data better than those tested above.

Discussion

Our results suggest a complex introduction history of *A. longifolia* around the world during the 19th and 20th centuries. We found support for our initial hypothesis that invasive populations have high genetic diversity and low population structure. This agrees with the known history of multiple introductions, often of large propagule sizes, of the species into many parts of the world (Kull et al. 2007, 2008, 2011; Carruthers et al. 2011). Multiple introductions, especially when originating from multiple sources, hamper inferences of the native sources of invasive populations, even when historical records are available. For example, for Portugal, we were unable to identify the native source(s) of introduction and found evidence of multiple introductions, thus refuting the single introduction hypothesis from a previous study (Vicente et al. 2018). Similarly, we were unable to draw any conclusions on the sources of invasive *A. longifolia* in Galicia (Spain), likely due to the low number of populations and individuals we sampled in the country. Regarding South Africa, as expected, we were not able to identify the native source of the invasion. On the other hand, we were able to identify Tasmania as the likely native source of invasive populations in South America. Below we discuss how our limited sampling in Victoria and South Australia (one population in each state) and lack of samples from Queensland may have impacted our inferences of the introduction histories of *A. longifolia* to different parts of the world.

We identified population structure in Australian *A. longifolia*, with populations from mainland Australia and Tasmania corresponding to two distinct genetic clusters (Figs 6, 7A). Most invasive populations appeared to be admixtures between these two clusters, providing support for multiple introductions. One possible explanation for the existence of the two genetic clusters in Australia is the Bass Strait that separates Tasmania from the mainland, and thus acts as a strong barrier to gene flow as has been observed for *A. dealbata* (Hirsch et al. 2017, 2018) and other Australian natives such as *Eucalyptus regnans* (Nevill et al. 2010) and *Tasmannia lanceolata* (Worth et al. 2010). We also identified substructure within the mainland Australia and Tasmania clusters. Within mainland Australia, the Beachport population is genetically distinct from all others (Fig. 7B), and this result is also corroborated by the cpSSR data. This population is from South Australia and corresponds to the most western sampling site in our analyses, and thus these results might be due to disjunct sampling. Future work should include populations sampled throughout this state to fully understand the species' population structure in Australia. Regarding the Tasmanian cluster, the Bridport, Three Sisters National Park, and Stanley populations were found to be genetically distinct from all others (Fig. 7C). Interestingly, these are the only three sampled Tasmanian populations in close proximity to the Bass Strait, and these results can possibly suggest some level of gene flow among these populations and those from mainland Australia close to the Bass Strait. However, only one sampled population in mainland Australia is situated within this region (Curdievale, Victoria), thus further sampling is required to clarify these genetic relationships.

While we identified substructure within mainland Australia and Tasmania, we used the two overall genetic clusters as putative source areas in our ABC modelling for several reasons. First, the overall clustering of Australian populations into two genetic clusters was well supported by our isolation by distance (IBD) analyses and regional comparisons of fixation indices (Fig. 8). Second, fine-scale source inferences using hierarchical clusters would be more affected by unsampled "ghost" populations (which we included in our modelling scenarios, Fig. 3) than inferences based on broadscale regional genetic clusters. Our limited sampling in several Australian states makes the issues associated with modelling fine-scale invasion sources even more problematic. Lastly, co-evolutionary diversification between the host plants and prospective biocontrol agents is generally evident at broader, rather than narrow, geographic scales (e.g., Goolsby et al. 2006). The earleaf wattle example we discussed earlier provides a case in point (see Introduction). Co-diversification between this wattle and its specialist leaf-feeding beetle, *Calomela intemerata*, has been identified over broad geographic scales (Nawaz et al. 2021). Therefore, knowledge of the broadscale native range source(s) of invasive populations can play an important role in matching potential biological control agent biotypes with invasive host plant lineages.

We also found evidence for a unique genetic cluster shared between Portugal and South Africa. Invasive populations in these two countries also shared one cpSSR haplotype (Fig. 4). These findings suggest a possible secondary introduction event or bridgehead, likely from South Africa into Portugal based on historical occurrence records of the species in both countries (Rei 1925; Lourenço 2009; Poynton 2009; Marchante 2011; Vicente 2016; Vicente et al. 2018). Our ABC modelling did not support this scenario and this aspect of the introduction history of *A. longifolia* warrants further investigation. Overall, globally invasive populations do not appear to have gone through genetic bottlenecks upon introduction, a common feature of invasive acacia populations (Vicente et al. 2021).

While we could not infer introduction histories for all invaded regions with high levels of confidence, we do provide evidence that these likely differed among different parts of the world. For instance, we found higher cpSSR haplotype diversity in South Africa and Portugal compared with South America, probably because these two countries have been invaded for longer or, more likely, their introductions had much higher propagule pressure than to those into South America. South Africa had the highest haplotype diversity (higher than in the native range populations we sampled), while Spain and Brazil had the lowest. These findings agree with historical records indicating that wattle seeds, including those of *A. longifolia*, were often imported into South Africa from several locations, both from within and outside Australia (Poynton 2009). Our finding that South African populations harbour genetic diversity levels similar to those in native range populations is therefore unsurprising. Taken together, this corroborates our Approximate Bayesian Computation (ABC) modelling inferences, which failed to identify the native source of South African populations based on the scenarios we analysed. Similar findings have been made for other invasive acacias with complicated introduction histories characterised by multiple introductions of high propagule sizes

from diverse sources (e.g., *A. dealbata*; Hirsch et al. 2019, 2021). In our case, it is also possible that the "ghost" sources of South African invasions are areas in Victoria, South Australia, or Queensland, from where we had limited or no samples, and this limitation may also be the reason for the identification of divergent chloroplast haplotypes in invaded areas (e.g., haplotypes A and H in South Africa, Fig. 4).

The introduction history of *A. longifolia* in Portugal, as for South Africa, is likely characterised by multiple and genetically diverse introduction events. Historical records indicate that various *Acacia* species were planted along the Portuguese coast at different times (e.g., Rei 1925; Lourenço 2009; Marchante 2011; Marchante et al. 2011; Vicente 2016; Vicente et al. 2018). A previous genetic study hypothesised that the same seed allotment was used to establish nursery stock from which plants were sourced for plantings along the Portuguese coast (Vicente et al. 2018). Yet, genetic diversity of Portuguese populations is similar to that in South African and Australian populations, suggesting that multiple introductions from different origins likely established invasive populations in Portugal. A similar introduction history was recently described for *A. dealbata* into Portugal (Hirsch et al. 2021). Again, the sources of Portuguese *A. longifolia* invasions may include native range areas in Victoria, South Australia, or Queensland, from where we had limited or no samples.

For Brazil and Uruguay our ABC analyses indicated that the most likely origin of introduction is Tasmania, either as single or multiple introduction events. Historical records from these countries are scarce but considering that the genetic diversity of these populations is similar to that found in South African, Portuguese and Australian populations, multiple introductions seem likely. We could not conclusively infer the source(s) of Spanish *A. longifolia* populations, mostly likely because of the low number of plants we sampled in this country. However, we speculate that these plants were introduced in similar fashion to that of Portugal.

Conclusion

Our work shows that the origins of *A. longifolia* introductions around the world are hard to trace, likely because of the extensive historical efforts to introduce the species for dune 'restoration' and as an ornamental plant (Kull et al. 2011). The similar levels of genetic diversity among native and invasive ranges are illustrative of this. Multiple introduction events of large size (i.e., high propagule pressure) will not only help introduced population to overcome demographic and stochastic impacts associated with small populations sizes but will also provide high adaptive capacity, afforded by high genetic diversity, to these populations. Our results showing extensive admixture between native range genetic clusters also suggest that exploration for new biocontrol agents can be done throughout the native range of *A. longifolia* as no 'pure' genetic lineages are invasive. Moreover, the success of existing biocontrol agents such as *Trichilogaster acaciaelongifoliae* in places such as South Africa is likely to be replicated in other invaded regions. 'Piggy-backing' on the biocontrol programs of countries such

as South Africa may substantially shorten the amount of time needed to implement programs in other parts of the world, as was the case for the introduction of *T. acaciae-longifoliae* into Portugal (e.g., Marchante et al. 2011; López-Núñez et al. 2021).

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All authors were involved in the research conceptualisation, data interpretation and in writing, reviewing, and editing the manuscript. SV, CM and JLR collected the samples. SV performed the laboratory work and data analysis.

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Supplementary material 1

Supplementary methodology details (primers, PCR conditions) and data analyses.

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Data type: Figures and tables

Explanation note: Comparison of the A) global fixation indices over all loci and populations of *Acacia longifolia*, and B) pairwise fixation indices, with and without the ENA correction (Chapuis and Estoup 2007). Comparisons of A) expected heterozygosity, B) observed heterozygosity, C) allelic richness, and D) inbreeding coefficent, using uncorrected and null allele-corrected datasets (Micro-Checker v2.2; Van Oosterhout et al. 2004). Box plots showing comparisons of expected heterozygosity (A), observed heterozygosity (B), allelic richness (C) and inbreeding coefficient (D) among *Acacia longifolia* populations from invaded countries (with populations from Portugal and Spain combined as Iberian Peninsula, IBP) and the two native range clusters identified by STRUCTURE analysis. Likelihood distribution [LnP(*K*)] and Delta *K* plots (Evanno method; Evanno et al. 2005) from STRUCTURE HARVESTER analysis for A-B) the complete dataset, and C-D) further analysis of the largest cluster (indicated in blue in Fig. 6A in the main text) identified in the analysis of the complete dataset (hierarchical analysis), respectively. STRUCTURE bar plots for $K = 3 – 6$ from the hierarchical analysis of the largest cluster from analysis of the complete dataset (indicated in blue in Fig. 6A in the main text). Results from the STRUCTURE HARVESTER analysis of the native range dataset. STRUCTURE bar plots for $K = 3 – 6$ from the analysis of the native range data only. Likelihood distribution [LnP(*K*)] and Delta *K* plots (Evanno method; Evanno et al. 2005) from STRUCTURE HARVESTER analysis for A-B) mainland Australia data, and C-D) Tasmania data, respectively. STRUCTURE bar plots for $K = 2 – 6$ (except $K = 4$) from the analysis of the mainland Australia data only. STRUCTURE bar plots for $K = 3 – 6$ from the analysis of the Tasmania data

only. Posterior probabilities of the six tested scenarios by invaded country. Details of microsatellite primers tested for cross-amplification in *Acacia longifolia*. Primers selected for cpSSRs and SSRs analyses and their corresponding repeat motifs, identified alleles, fluorescent dye labels used, PCR conditions (annealing temperature and concentration of magnesium chloride). Descriptions of parameters included in the ABC analyses. Prior and posterior values of parameters for Scenario 6 of ABC analyses, selected as the best scenario for Portugal (POR) and South Africa (RSA). Prior and posterior values of parameters for A) Scenario 2 and B) Scenario 5 of ABC analyses, selected as the best scenarios for Brazil (BRA) and Uruguay (URU). Type I and Type II errors for the chosen scenarios of each invaded country in ABC analyses. Results of the "model checking" analysis for the best scenarios selected by country in ABC analyses. Posterior probabilities of the six bridgehead tested scenarios between RSA and POR (600 000 simulations in total). DIYABC drawing of scenario 5, which had the higher posterior probability of the six bridgehead tested scenarios between RSA and POR (p = 0.7161, 95% CI = 0.6165-0.8157). Results of the "model checking" analysis for the best bridgehead scenarios selected (scenario 5) in ABC analyses.

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Supplementary material 2

Genotype data used in this study in GenAlEx format.

Authors: Sara Vicente, Helena Trindade, Cristina Máguas, Johannes J. Le Roux

Data type: Genotype data

Link: https://doi.org/10.3897/neobiota.82.87455.suppl2

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REVIEW ARTICLE

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Shining a LAMP on the applications of isothermal amplification for monitoring environmental biosecurity

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Abstract

Environmental biosecurity risks associated with the transnational wildlife trade include the loss of biodiversity, threats to public health, and the proliferation of invasive alien species. To assist enforcement agencies in identifying species either intentionally (trafficked) or unintentionally (stowaway) entrained in the trade-chain pathway, rapid forensic techniques are needed to enable their detection from DNA samples when physical identification is not possible. Loop Mediated Isothermal Amplification (LAMP) is an emerging technique, with recent applications in biosecurity and forensic sciences, which has potential to function as a field-based detection tool. Here we provide an overview of current research that applies LAMP to environmental biosecurity, including identification of ornamental wildlife parts, consumer products, and invasive species monitoring and biosecurity detection. We discuss the current scope of LAMP as applied to various wildlife trade scenarios and biosecurity checkpoint monitoring, highlight the specificity, sensitivity, and robustness for these applications, and review the potential utility of LAMP for rapid field-based detection at biosecurity checkpoints. Based on our assessment of the literature we recommend broader interest, research uptake, and investment in LAMP as an appropriate field-based species detection method for a wide range of environmental biosecurity scenarios.

Keywords

Environmental biosecurity, invasive species, loop mediated isothermal amplification (LAMP), wildlife forensics

Introduction

A primary biosecurity concern is the early detection and accurate identification of novel invasive species (Early et al. 2016) and diseases (Bezerra-Santos et al. 2021). The costs of managing invasive species globally since 1960 are at least $95 billion (Cuthbert et al. 2022), with the damages and losses caused being at least a magnitude greater (Cuthbert et al. 2022). Yet proactive prevention measures accounted for only *c*. 3% of the management cost (Cuthbert et al. 2022). This indicated a strong priority in most countries for spending on post-establishment control and eradication, despite the obvious benefit of preventative management, including effective interception and detection measures (Cuthbert et al. 2022). As such, strong and effective environmental biosecurity measures are required, here defined as the protection of the environmental and social amenity from the negative impacts of invasive species (DCCEEW 2022). Environmental biosecurity spans the whole biosecurity continuum, which includes pre-border preparedness, border protection and post border management and control.

The implications of poor biosecurity management extend to biodiversity loss, which is well documented for source populations when species are subject to illicit trade (Morton et al. 2021). Wildlife trade can additionally incur a biosecurity threat when species with high invasion potential are introduced and establish in novel ecosystems resulting in a loss of ecosystem services (Charles and Dukes 2007). The intersection of biodiversity loss and biosecurity is often realised as a consequence of ongoing globalisation and transnational trade in live animals (stowaways (Hulme 2021) or pets (Lockwood et al. 2019) and wildlife products (food, medicines and ornaments (Ege et al. 2020)). Limiting the impacts of biodiversity loss is a key biosecurity goal which spans all points along the biosecurity continuum (Outhwaite 2010).

Within environmental biosecurity several molecular biomonitoring techniques have gained prominence. These techniques include DNA barcoding (Armstrong and Ball 2005), with recent advancements including DNA barcode sequencing at border checkpoints (Abeynayake et al. 2021), species-specific TaqMan assays leveraging portable thermocycling technologies (Trujillo-González et al. 2022), and metabarcoding approaches in ports (Borrell et al. 2017; Grey et al. 2018). To achieve reliable biosecurity intervention, low-cost, low-resource, rapid forms of species detection and identification are required. Lengthy analysis can result in delayed legal action with substantial resource-based costs, including long turnaround times (Masters et al. 2019). Within the literature there is an increasing emphasis on the benefits of cross disciplinary collaboration, and research to aid in the development of field-ready technologies to address these limitations and increase rapid species detection (Masters et al. 2019; Smith et al. 2019). Biosecurity is one such field that has recently embraced the advent of new technologies including portable thermocyclers and recent isothermal amplification to tackle rapid onsite (defined as the point of interception) detection of emerging threats.

The application of isothermal amplification methods for onsite monitoring of non-native species crossing transnational borders has been explored (Kyei-Poku et al. 2020; Vythalingam et al. 2021); as they offer an operational tool well suited for highly sensi-

tive and specific field-based detection (Figure 1). Here, we critically examine the novel applications of isothermal amplification methods such as Loop Mediated Isothermal Amplification (LAMP) (Yu et al. 2019) and Recombinase Polymerase Amplification (RPA) (Hsu et al. 2021) for biosecurity detection of invasive alien species, with a particular focus on animals. We highlight the benefits for onsite detection and discuss research that has explored this tool for wildlife forensic science, biosecurity, and interrelated fields. We discuss emerging technologies and the future direction of LAMP, when applied to field-based detection. Notably, we recommend broader interest, greater research uptake, and further investment in LAMP as an appropriate field-based species detection method for a wide range of environmental biosecurity scenarios.

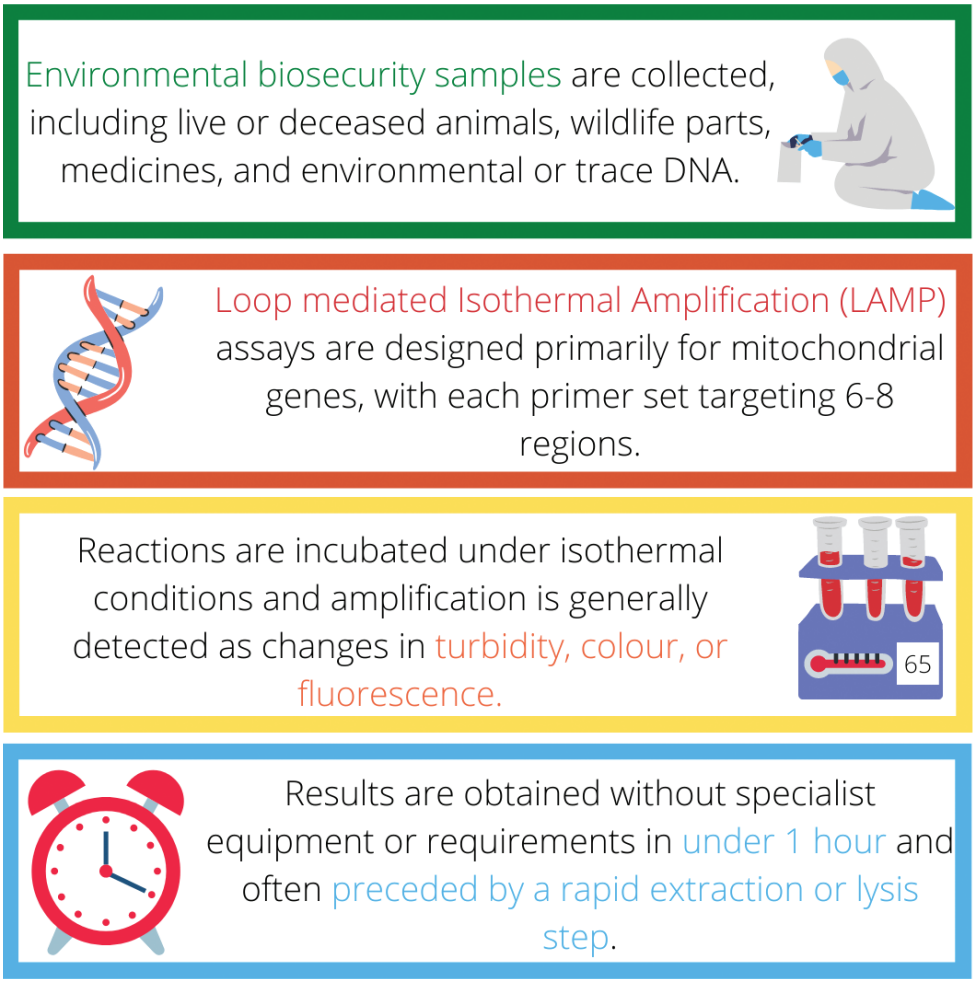

Figure 1. Workflow indicating the integration of Loop Mediated Isothermal Amplification (LAMP) into an environmental biosecurity scenario. This generally requires appropriate sample collection and storage, *in silico* primer design and validation, isothermal incubation conditions with detection facilitated by changes in turbidity, colour or fluorescence. LAMP reactions often lead to positive detection in under 1 hour without requiring specialist equipment.

Loop Mediated Isothermal Amplification (LAMP)

LAMP is a nucleotide amplification method that functions by auto-cycling strand displacement DNA synthesis, performed by a DNA polymerase with high strand displacement affinity (Notomi et al. 2000; Nagamine et al. 2002). This method combines rapid, simple, and highly specific target sequence amplification (Notomi et al. 2015). LAMP utilises two inner and two outer primers with the option of additional loop primers that together recognise six to eight distinct regions on the target DNA, facilitating high specificity (Nagamine et al. 2002; Tomita et al. 2008). The LAMP technique can amplify a few copies of DNA exponentially in less than one hour. The reaction process consists of two forms of elongation occurring via a loop region. This includes template self-elongation starting at the stem loop formed at the 3'-terminal end and subsequent binding and elongation of new primers to the loop region (Figure 2) (Notomi et al. 2015). In addition to target specificity, primary advantages include the speed and simplicity of the reaction, which is conducted at a single (isothermal) reaction temperature (Francois et al. 2011). This reduces the need for sequential thermocycling stages and the associated expensive and specialised thermocycling equipment, most often restricted to a dedicated laboratory (Francois et al. 2011). LAMP has additionally shown tolerance to PCR inhibitors, pH and temperature variability (Francois et al. 2011).

LAMP is versatile, as detection methods can be divided into three primary categories including turbidity, fluorescence, or colorimetric. Initially detection was measured as a change in turbidity visible due to white by-product precipitation of magnesium pyrophosphate in the reaction mixture (Mori et al. 2001). This is possible as both an endpoint and real-time measurement, as the production of precipitate correlates with the amount of DNA synthesised (Mori et al. 2004). In terms of fluorescence detection several studies indicated the use of intercalating fluorescent dyes, including SYBR green I (Kumari et al. 2019) and melting and annealing curve analysis post real-time monitoring (Cho et al. 2014). Additionally, results of the LAMP reaction are often visualised as a unique banding pattern by gel electrophoresis (Chen et al. 2013), which may also serve as a confirmatory indicator of LAMP reaction success (Jackson et al. 2020). The use of colorimetric methods, particularly by use of additives such as hydroxy naphthol blue, phenol red, calcein, leuco crystal violet, and malachite green (Goto et al. 2009; Scott et al. 2020), are common and widespread in several applications and often depend on pH (Tanner et al. 2018). All three forms of detection can be monitored by eye at the endpoint of the reaction. However, innate subjectivity remains an issue, and as such, turbidimeters and fluorometers are often used to facilitate quantitative measures of the LAMP reaction (Zhang et al. 2014). Concerning colorimetric methods, LAMP detection is often accompanied by optimised imaging procedures (Rodriguez-Manzano et al. 2016) or software to eliminate innate colour subjectivity. In some cases, open source (e.g., ImageJ (Schneider et al. 2012)) plugins have been developed to distinguish between negative and positive reactions based on colour components such as hue (Scott et al. 2020; Layne et al. 2021; Woolf et al. 2021). Additionally, the properties of colorimetric reactions can allow for conformation assessments by use of the UV-vis spectrum to observe the transition of colour altered peak intensities between positive and negative reactions (Nguyen et al. 2019a).

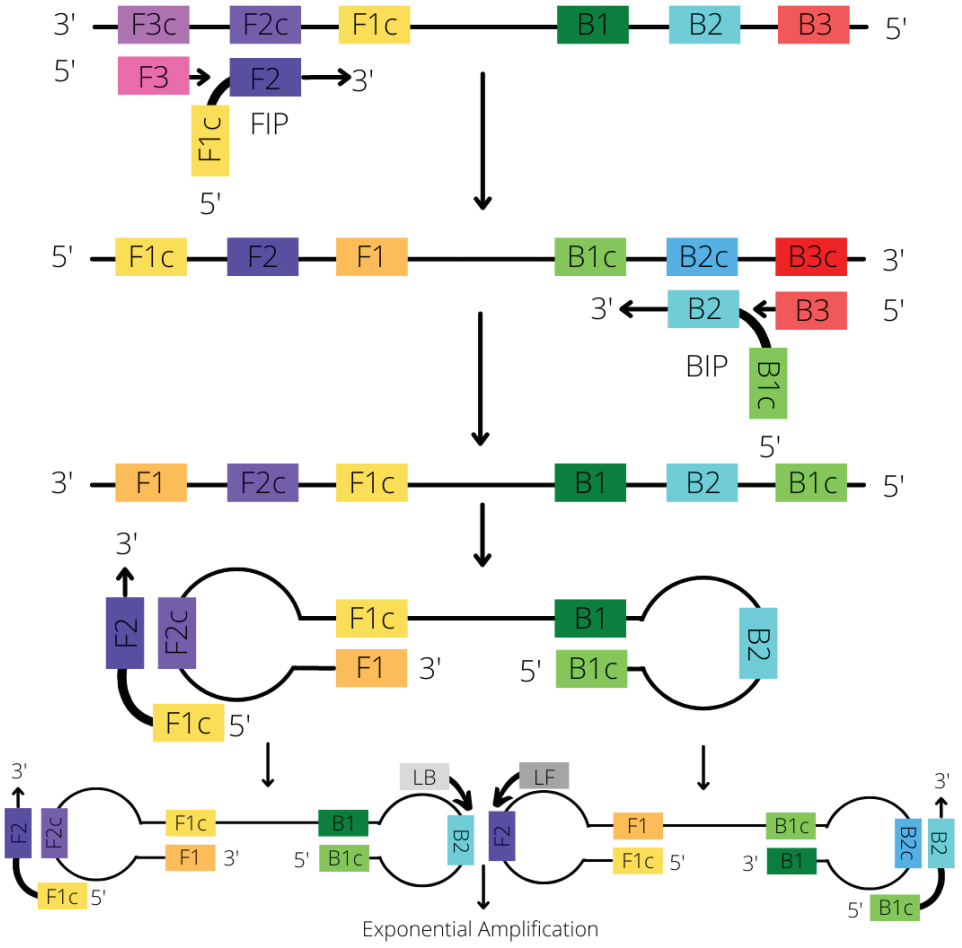

Figure 2. Loop mediated amplification mechanism. Two inner primers consisting of the F3 and forward inner primer (FIP) and two backward primers, the B3 and backward inner primer (BIP) are used to target 6 regions. Additionally, loop primers are often used to accelerate the reaction, denoted here as LF (loop forward) and LB (loop backward) targeting two additional distinct regions. The *Bst* polymerase displaces each of the DNA strands and initiates synthesis; this leads to the formation of loop structures, which facilitate subsequent rounds of amplification.

Until recently, the primary role of LAMP was to detect single targets with reasonably high specificity. The use of turbidimetric, colorimetric and fluorometric detection is often considered a form of indirect evaluation, functioning in a similar way to SYBR green qPCR assays (Liu et al. 2017b). The integration of molecular probes or beacons in LAMP research emerged as a means of reducing false positives due to non-specific amplification (Liu et al. 2017b; Hardinge and Murray 2019). One of the initial studies incorporated a quencher-fluorophore duplex region on LAMP primers aimed at expanding detection to multiple targets (Tanner et al. 2012). When primers anneal to the desired target the fluorophore is released and a gain of fluorescent

signal can be observed. This has been showcased for real time detection of 1–4 target sequences utilising a fluorometer to detect as few as 100 copies of human genomic DNA (Tanner et al. 2012). The molecular probe-based approach has facilitated greater specificity and unlocked multiplexing capacity. These methods have also diversified to include assimilating probes (Kubota et al. 2011), TaqMan coupled LAMP (Yu et al. 2021), fluorogenic bidirectional displacement probe-based real-time LAMP (Ding et al. 2016), locked nucleic acid molecular beacons (Bakthavathsalam et al. 2018) and self-quenching/de-quenching probes (Gadkar et al. 2018). Additionally, the role of primer dimer and self-amplifying hairpins on reverse transcription LAMP when detecting viral RNA has also been explored (Meagher et al. 2018). Minor displacements of primers to regions of self-complementarity away from the 3' end of the primer dramatically reduced the occurrence of secondary structures and improved speed and in some cases sensitivity (Meagher et al. 2018). Furthermore, mathematical models to identify non-specific amplification, distinguishing between target and non-target amplification based on microchip electrophoresis have also been developed (Schneider et al. 2019). Stoichiometric and pseudo kinetic modelling has also been conducted to classify LAMP products into uniquely identifiable categories, aimed at aiding robust probe-based detection strategies enhancing specificity (Kaur et al. 2020).

Table 1 lists the advantages and disadvantages of LAMP in contrast to PCR, which is the most generally applicable amplification method in environmental biosecurity (McGraw et al. 2011). However, sample type, equipment availability, DNA isolation and handling, downstream analysis and intended application will influence suitability. PCR methods, as applied to environmental biosecurity, are also rapidly developing to incorporate field detection using compact and mobile thermocyclers with integrated molecular probes, exemplified by comparisons conducted for TaqMan assays and LAMP assays (Trujillo-González et al. 2022). Perhaps the main advantage of LAMP in contrast to advancements in PCR, is the ease of integration due to the lack of thermocycling requirements. Denaturation is facilitated by a strand displacing polymerase, and amplification is conducted at a constant (*c.* 65 °C) incubation temperature (Nagamine et al. 2001). These key features drastically lower the equipment requirements to a simple heat block, pipettes and an appropriate sample handling space that facilitate the integration of this technique into onsite contexts without large financial burdens to the end-user.

Thus far the application of LAMP has primarily focused on cases in which high quality DNA is available from sample types such as tissue and whole specimens. However, many environmental biosecurity samples include degraded DNA (e.g. animal material that has been cooked, processed or treated with chemicals) or environmental DNA (e.g. faecal samples, swabs of empty containers, water, soil or air samples). LAMP could function sub-optimally in scenarios which commonly involve highly degraded template DNA due to the need for more than two primers and environmental samples that contain DNA from multiple sources. As such we recommend further research into LAMP suitability for a greater range of taxa and sample types including degraded DNA and environmental DNA subject to complex and varied environmental conditions as this influences DNA decay rates (Andruszkiewicz Allan et al. 2021).

Table 1. Advantages and disadvantages of LAMP compared to the most common amplification method in environmental biosecurity, the Polymerase Chain Reaction (PCR).

Method	Advantage	Disadvantage
LAMP	High specificity (4–6 primers), with probe capacity.	Challenging design parameters.
	No heat denaturation step required.	Degraded DNA may prevent primer annealing.
	Isothermal incubation, with low resource requirements.	Limited downstream applications for amplicons.
	Colorimetric, turbidimetric, fluorogenic real-time and endpoint detection capacity.	
	Speed of reaction (generally <60 minutes).	
	High tolerance to inhibitors commonly encountered in field samples.	
PCR	Widely available	Thermocycling required, including high temperature for separation of strands, to facilitate primer binding
	Downstream capacity, including sequencing.	Expensive thermocycling equipment required, often restricted to a dedicated laboratory space.
	Low cost of reagents and primer synthesis.	Lower specificity due to only two primers, however probes can be integrated but at much greater cost to the end-user.
		Visualisation of results requires fluorometer or gel electrophoresis.

LAMP research for environmental biosecurity applications

The most common application of DNA based detection in wildlife forensic science investigations is species detection and identification (Linacre 2021). As a result, LAMP has been applied to environmental biosecurity detection cases relating to: (1) identification of adulterated animal products (Cho et al. 2014; Liu et al. 2019; Nikunj and Vivek 2019; Sul et al. 2019); (2) detection of conservation significant species and disruption of illegal wildlife trade (Yu et al. 2019; But et al. 2020; Wimbles et al. 2021); and (3) biosecurity screening (Blaser et al. 2018b; Kyei-Poku et al. 2020), and detection of invasive species (Williams et al. 2017; Rizzo et al. 2021; Vythalingam et al. 2021); including disease monitoring (Sahoo et al. 2016). Additionally, LAMP has a strong presence in bacterial and viral point-of-care detection methods research (Nguyen et al. 2019b; Kashir and Yaqinuddin 2020).

(1) Falsified consumer items and product authenticity

Detection of falsified fur products has been explored using a highly specific fluorescence based LAMP assay targeting the *cytochrome oxidase subunit (CO1)* gene for both fox and cat fur (Yu et al. 2019). This assay was developed in response to environmental biosecurity concerns of illicit harvesting and subsequent commercial fraud. The assay is tolerant to inhibitors such as pigments, dyes, or other fur components, with the authors highlighting its potential role as an on-site species identity test, without costly requirements or specialist equipment (Yu et al. 2019). Sensitivity is similar to PCR, detecting down to 10 and 1 pg of DNA for cats and foxes respectively (Yu et al. 2019).

The detection of food products, which have been mislabelled, tampered, or contain mixed species material is of particular interest. Assays targeting the *16s rRNA* region have been developed to detect chicken from processed meat samples, in under 30 minutes, with a detection limit of 10 fg (Sul et al. 2019). Similarly, targeting the *cytochrome b* region, ostrich meat can be detected in mixtures constituting only 0.01% in as little as 15–20 minutes (Abdulmawjood et al. 2014), and pork with a detection limit of 1 pg without cross reactivity (Yang et al. 2014). Additionally, a LAMP assay targetting the mitochondrial D-loop region has been developed for cattle, tested on meat samples with a detection limit of 10 pg of DNA (Kumari et al. 2019). The underlying drivers behind this research interest are varied and include religious certification, and concerns relating to allergens (Mao et al. 2020; Sheu et al. 2018), fraud (Kumari et al. 2019), disease (Pang et al. 2018; Zhao et al. 2010) and identifying species of conservation significance (But et al. 2020).

LAMP assay development also extends to the seafood industry, including detection of jumbo flying squid from tissue samples, with a LAMP assay targeting *CO1* with a detection limit of 10 pg of DNA per reaction (Ye et al. 2017). Several studies focussed on the detection of mislabelled or falsified seafood products have integrated molecular beacons into LAMP assays, facilitating increased specificity. Two such studies utilise self-quenching fluorogenic probes targeting skipjack tuna (Xu et al. 2021) and Atlantic salmon (Li et al. 2022). An initial skipjack tuna LAMP assay utilised non-specific fluorescent dyes targeting the *cytochrome b* region relying primarily on the specificity of primer annealing for species-specific sequences (Xiong et al. 2021b). The integration of a self-quenching fluorogenic probe, attached to the FIP primer, facilitated skipjack tuna authentication from tissue samples, and decreased the likelihood of false-positive signals when assessing six commercial tuna products (Xu et al. 2021). This assay displayed exceptional sensitivity, detecting as little as 5 fg of skipjack tuna DNA (Xu et al. 2021). Similarly, an initial non-specific fluorescence based LAMP assay was developed for Atlantic salmon targeting a section of the *cytochrome b* (Xiong et al. 2021a), prior to integrating a self-quenching fluorogenic probe attached to the backward loop primer for identification of tissue samples with a detection limit of 5 pg (Li et al. 2022).

Highly specific, sensitive, and rapid detection of bushmeat samples is of considerable interest to conservation scientists and environmental biosecurity enforcement bodies, as trade in bushmeat is directly linked to biodiversity loss (Ripple et al. 2016) and emerging zoonotic disease (Hilderink and de Winter 2021). Therefore, research presented here could have similar implications for the detection of bushmeat-related wildlife crimes. Providing point-of-entry detection could facilitate greater biosecurity preparedness and decrease transnational incursions through wildlife crime interception. Genetic reference frameworks for African forest bushmeat have already been established (Gaubert et al. 2015) and could form the basis for LAMP onsite detection of transnational trafficking. This is particularly true when identifying bushmeat for species covered by national or international protections as conducted for the Cameroonian bushmeat trade, where >50% of bushmeat species traded were nationally protected (Din Dipita et al. 2022). Nearly half of all samples collected from the Cameroonian bushmeat trade,

subject to morphological identification, were corrected when subject to DNA based analysis, with additional high rates of incorrect identification at Parisian customs (Din Dipita et al. 2022). This further illustrates the need for highly specific, rapid forms of species identification based on LAMP, operationalised for a field environment.

(2) Illegal wildlife trade and conservation monitoring

LAMP has been showcased for field-based detection of illegal trade in shark fin products, which can be directly applied to enforcing environmental biosecurity regulations and CITES obligations; as rapid LAMP detection has been developed for twelve CITES-listed shark species (But et al. 2020). The assays include primers that target the *CO1* and *NADH2* sequences and can detect all twelve species individually from tissue sample within an hour at constant temperatures (But et al. 2020). The cost of each LAMP reaction was *c.* US$0.6 compared with *c.* US$0.25 for a comparable PCR workflow, with the advantages of LAMP primarily spanning field applicability and high specificity (But et al. 2020). This study presented a novel application of LAMP onsite checkpoint monitoring for species with high wildlife crime concern. Similar methods could be explored for the rapid identification of other endangered species including those common in the illegal pet trades. This is also true for current wildlife forensic methods that employ PCR, as they could benefit from LAMP based presumptive testing prior to laboratory validation, reducing the number of samples requiring exhaustive laboratory-based testing.

The mutual benefits of field-based LAMP monitoring for conservation and the prevention of wildlife crime have been realised for combatting cases of wildlife poaching, specifically for the white rhinoceros (Wimbles et al. 2021). Rhinoceros horn is a commodity common in illegal transnational marketplaces (Hübschle 2016); the consequent nefarious trade has received widespread wildlife forensic attention (Ewart et al. 2018a; Ewart et al. 2018b). The internationally standardised rhinoceros horn identification test is PCR-based and as such has limited applicability to onsite detection outside a laboratory setting. Wimbles et al. (2021) presented a white rhinoceros specific LAMP assay, targeting the *cytochrome b* region, integrated into a microfluidic device capable of field-based detection in 30 minutes from dung samples, including field testing carried out at the Knowsley Safari; the approach could similarly play a role in the detection of wildlife crimes. The microfluidic device presented by Wimbles et al. 2021 included DNA extraction followed by three wash chambers prior to LAMP, with positive and negative control chambers adjacent to the field sample chamber for confirmation of positive detection. This study highlighted the possibility of LAMP microfluidic devices to operate in a myriad of wildlife crime situations, offering rapid, cost-effective, portable presumptive genetic testing.

Other forms of wildlife crime, including additional cases of poaching (Kumar et al. 2012; Ghosh et al. 2019) and trafficking of wildlife parts (Gupta 2018), could also benefit from on-site presumptive detection. This is particularly true for situations in which the sample itself bears insufficient physical characteristics or on-site detection

to species level is time sensitive. Onsite identification has been showcased for a species susceptible to illegal hunting, the Formosan Reeves' Muntjac (Hsu et al. 2021). An RPA assay has been developed for the isothermal detection of bush meat in combination with a lateral flow strip. The described assay targeted the *cytochrome b* gene region and detected the target species from extraction to result in around 30 minutes. As such, the application of isothermal amplification methods to the detection of a range of wildlife crimes seems well suited, particularly when indistinguishable tissue samples are the only form of remaining evidence.

(3) Invasive species monitoring

Monitoring and related control programs have recently focussed on the role of eDNA in invasive species detection (Hunter et al. 2015; Morisette et al. 2021), with several studies focusing on LAMP as a potential eDNA monitoring tool (Williams et al. 2017; Vythalingam et al. 2021). The emphasis on monitoring primarily concerns invertebrate pests, largely when all or part of the organism is available, as demonstrated by the development of LAMP assays for point of entry detection (Blaser et al. 2018a).

Border surveillance of emerging insect incursions

A range of LAMP assays have been developed for multiple insect species commonly of environmental biosecurity concern (Table 2). This primarily concerns stowaways, with some assays developed as early warning tools for incursion events (Kyei-Poku et al. 2020). In addition to early detection, studies have tested detection in mixed samples, including red fire ants (Nakajima et al. 2019). Red fire ants are classed as a super pest with introductions as stowaways linked to early global trade routes (Gotzek et al. 2015); continued interest in their further spread throughout Australia and Asia demands robust biosecurity testing (Wylie et al. 2020). Another horticultural focus is the detection of fruit fly species (Huang et al. 2009; Blaser et al. 2018a; Blaser et al. 2018b; Sabahi et al. 2018). One study focussed on the detection of several regulated quarantine insects at Swiss borders, which included fruit fly genera *Bactrocera* and *Zeugodacus* (Blaser et al. 2018a). Several primer sets targeting *CO1* were used to detect fruit fly and *Bemisia tabaci*, *Thrips palmi*, which are two additional species of biosecurity concern (Blaser et al. 2018a). Laboratory evaluations of the developed assays for 282 specimens suspected to be invasive, indicated a 99% test efficiency in under 1 hour (Blaser et al. 2018a). Several studies have focused on the detection of fall armyworm (Agarwal et al. 2022; Congdon et al. 2021; Kim et al. 2021). The most recent is based on the *CO1* gene, with high specificity and sensitivity down to 2.4 pg of DNA (Agarwal et al. 2022). Furthermore, the study contrasts previous work (Kim et al. 2021) conducted for a *tRNA* based LAMP assay indicating the time-based advantage of added loop primers for the described *CO1* assay (c. 10 mins to result) (Agarwal et al. 2022).

Additionally, a LAMP assay has been developed for Khapra beetle targeting the *16s rRNA* region with an additional LAMP assay targeting the *18s rRNA* region used

Table 2. Summarised LAMP assays as applied to environmental biosecurity of high-risk insects. Includes the species name, the gene that the LAMP primers target, the tested sample types, the detection limit tested in the described study, time to detection and source. Fields containing 'not applicable' (N/A) are those for which detection limit wasn't tested directly or a different measure of sensitivity was used.

Species	Target	Tested sample types	Limit of detection	Time to detection	Source
Emerald ash borer	*CO1*	Adults, larvae, eggs, larval frass	0.1 ng	30 min	(Kyei-Poku et al. 2020)
Red fire ant	*CO1*	Whole specimen	N/A	90 min	(Nakajima et al. 2019)
Species belong to genera *Bactrocera* and *Zeugodacus* and *Bemisia tabaci* and *Thrips palmi*	*CO1*	Adults, larvae, and 1 mm^3 of larval tissue	N/A	60 min	(Blaser et al. 2018a)
Aedes mosquito species	*ITS1* and *ITS2*	Adult or larval stage specimen and eggs	N/A	60 min	(Schenkel et al. 2019)
Walnut twig beetle	28S rRNA	Adults and frass	1.3 pg and 6.4 pg for adults and frass, respectively	<30 min	(Rizzo et al. 2021)
Fall army worm	*CO1*	Adult and larval specimen	2.4 pg	<20 min	(Agarwal et al. 2022)
Fall army worm	*CO1*	Larvae	24 pg	<30 min	(Congdon et al. 2021)
Fall army worm	*tRNA* coding region between *ND3,* and *ND5*	Larval tissue	10 pg	90 min	(Kim et al. 2021)
Khapra Beetle	18s rRNA	Adults and larvae	1 fg	<25 min	(Rako et al. 2021)
New Guinea fruit fly	*CO1, EIF3L*	Tissue (3 fly legs)	10 copies for COI and 1000 copies for BtrivEIF3L	<25 min	(Starkie et al. 2022)

to detect the presence of interspecific beetle DNA (Rako et al. 2021). The Khapra LAMP assay had a limit of detection comparable to the Khapra real-time PCR test with a detection limit of 1.02 fg (Rako et al. 2021). This assay was assessed for extracts from Khapra beetle tissue samples using both laboratory-based, destructive, and crude extraction methods. A subsequent comparative study assessed the utility of this Khapra LAMP assay against two Khapra beetle specific TaqMan PCR assays for onsite biosecurity for samples collected from airborne and floor dust (Trujillo-González et al. 2022). Notably, extracted Khapra beetle eDNA from dust samples was amplified by qPCR, but not using the LAMP assay (Trujillo-González et al. 2022). A potential reason for the discrepancy between amplification methods could be the use of six primers, which may not all anneal to desired template DNA in situations with degraded DNA (Trujillo-González et al. 2022). These results highlighted an important consideration for LAMP application to environmental biosecurity, primarily sample types and end user application prior and throughout the assay development. LAMP assays may thus function best in environmental biosecurity scenarios from which high-quality DNA can be acquired, offering rapid presumptive species level testing.

Invasive aquatic species detection

A primary issue concerning biosecurity is the role that transnational trade in exotic pets can play as a source of invasive species, documented by the pet release pathway (Sinclair et al. 2020). Pet releases are often a driver of invasive species introductions (Liang et al. 2006; Lockwood et al. 2019), with invasiveness positively associated with commercial success of pets in trade (Gippet and Bertelsmeier 2021). Additionally, the import and export of pets is often highly regulated or strictly banned under national jurisdictional law (Ege et al. 2020). This has led to the development of detection methods for common aquatic pet species that double as invasive species in Malaysian waterways (Vythalingam et al. 2021). The focus species included guppies, goldfish, siamese fighting fish, Amazon sailfin catfish, koi and African sharptooth catfish, which were collected from local aquariums and pet shops for the purpose of LAMP development. DNA was extracted from caudal fin cuttings, with dilutions used to assess limits of detection. The resulting highly sensitive assays utilised 5 separate species-specific primer sets with a detection limit of between 0.02 pg and 2×10^{-12} pg for all 5 species (Vythalingam et al. 2021). The aim of the developed assay is to aid authorities in handling monitoring programs by providing rapid identification of non-native fish in ecosystems. Coupling this technique with optimal environmental DNA sampling has great potential for onsite monitoring. As such, programs tackling ecosystem monitoring for invasive species could benefit from assays targeting a wider range of invasive species. It is generally agreed that prevention is preferable to control of an established pest (Leung et al. 2002), as such investment in appropriate on-site LAMP detection could be paramount in preventing novel introductions.

Confirming the presence of aquatic pest species has been explored through the development of LAMP based assays for monitoring quagga and zebra mussels in river basins (Williams et al. 2017; Carvalho et al. 2021). The first study addressing LAMP development aimed at streamlining the eDNA detection of quagga and zebra mussels in Michigan lakes (Williams et al. 2017). This included the development of three LAMP assays, one targetting the *18s rRNA* gene, amplifiying both target species DNA with a detection limit of 0.0001 pg. A further two *CO1* assays targetted quagga and zebra mussels seperately, with a sensitivity of 0.001 pg and 0.01 pg respectively (Williams et al. 2017). Sample types included grab and concentrated surface water samples, containing both free DNA as well as larger cells and particulates, such as veligers, eggs, or seeds (Williams et al. 2017). These sample types were subject to direct amplification without DNA extraction, illustrating the tolerance of LAMP to environmental inhibitors (Williams et al. 2017). A subsequent novel zebra mussel assay targetted the *CO1* gene with a detection limit of 1.12 pg when tested against meat and water samples, which was also developed and field tested for a range of sample types collected from Portuguese, Spanish and French sources (Carvalho et al. 2021). An additional application has been the delimitation of eels in the genus *Anguilla*, with a focus on *Anguilla anguilla*, a critically endangered species (Spielmann et al. 2019). This assay was developed as a detection method for introduced foreign eel species in European rivers, protecting consumers against mislabelled eel consumables and could serve a role in ecological studies (Spiel-

mann et al. 2019). One LAMP assay was developed to detect all *Anguilla* species targetting the *C-type lectin* gene, while another targetted the mitochodrial D-loop region of *A. anguilla* with high specificity; both assays had a limit of detection of 500 pg (Spielmann et al. 2019). Sample types included muscle tissue of smoked processed fish and single eggs from *A. aguilla* as a proxy for on-site detection (Spielmann et al. 2019).

Current conventional aquatic eDNA monitoring methods are rapidly developing, including integration of qPCR assays with specialised eDNA sampling methods and miniaturised infield thermocyclers (Thomas et al. 2020). LAMP assays can function either in parallel to current best practise qPCR methods or in low resource contexts where isothermal end-point reaction conditions are most suitable. LAMP assays developed to target Quagga and Zebra mussels, are some of the few assays which have been tested against samples subject to environmental conditions and indicated tolerance and suitability within their respective contexts (Williams et al. 2017; Carvalho et al. 2021). The use of LAMP as an emerging surveillance technique for biosecurity officers and wildlife managers could thus be instrumental for conducting routine monitoring programs and detecting high risk environmental biosecurity threats.

Health and disease: detection and prevention

An often-overlooked component of environmental biosecurity is the potential introduction of foreign or novel wildlife diseases or zoonoses (Smith et al. 2012). An influx in zoonotic disease research brought on by the COVID-19 pandemic has highlighted the role of wildlife trade in the emergence of zoonotic diseases (Hilderink and de Winter 2021). Consequently, LAMP-based detection could be highly suitable to the detection of domestic and wildlife related introductions of novel diseases, strengthening environmental biosecurity. There are, in fact, a myriad of studies that explore LAMP based detection of COVID-19 to address on-site testing capacity of this global health concern (Augustine et al. 2020; Kashir and Yaqinuddin 2020; Dewhurst et al. 2022). Extending this research to a broader range of emerging pathogens and hosts could prevent future outbreaks and curb pandemics.

He et al. (2022) has presented multiple threatening pathogens, which are hosted by wild animals prized as delicacies in the Chinese Illegal Wildlife Trade (IWT). When 1941 animals from five mammalian orders were surveyed, 102 mammalian infecting viruses were discovered with 21 of those posing potential risk to humans (He et al. 2022). Among the species that had their virome characterised was the Raccoon dog. This species was identified as carrying a range of novel pathogens (He et al. 2022), including previous detections of close relatives of SARS-CoV and SARS-CoV-2 (Guan et al. 2003) and *Rotavirus A* (Abe et al. 2010). Raccoon dog meat is often used as a subsidiary component in meat mixtures, with reports of health deterioration in some consumers (Liu et al. 2017a). Consequently, a LAMP assay targeting *cytochrome b* has been developed to detect Raccoon dog in processed meat, indicating no cross reactivity with seven non-target species and target DNA detection limits of 0.2 pg (Liu et al. 2017a). These results indicated a demand for the detection of species common in the

IWT and present an opportunity for multiplex LAMP assays targeting both pathogens and hosts in tandem.

Detection of other zoonotic diseases has also gained some traction with the development of a LAMP assay for *Leptospira* (Chen et al. 2016). Leptospirosis is one of the most widespread zoonosis and is caused by a pathogen that colonises the renal tubules of hosts such as dogs, rats, and cattle (Chen et al. 2016). The *Leptospira* LAMP assay, targeting the *lipL32* and *lipL41* genes, offers exceptional sensitivity with a detection limit of 12 DNA copies. Additionally, LAMP reagents were lyophilised and stored, remaining stable for as long as 3 months at 4 °C (Chen et al. 2016). Storage and shelf life are additional considerations that are often omitted from publications concerning field-ready LAMP. These are, however, conditions that will have major impacts on field suitability and should thus be assessed.

Salmonella is considered a major food borne pathogen globally, which is responsible for food contamination leading to food poisoning (Zhao et al. 2010). As such, a myriad of LAMP assays and related methodologies have been devised for rapid point-of-care detection (Zhao et al. 2010). Initial studies developed assays targeting the genus specific *InvA* target that could detect 214 strains in 45 minutes with a detection limit of 1 pg of DNA (Zhao et al. 2010). Assessment of LAMP robustness has also been conducted for *Salmonella enterica* serovar Typhi, indicating consistency across two pH units (7.3–9.3) and temperatures of 57–67 °C with maintained specificity (Francois et al. 2011). This has since progressed with the integration of molecular probes (Mashooq et al. 2016), development of a related handheld device for the detection of *Salmonella enterica* (Jenkins et al. 2011) and integration of disk-based compact micro-reactors for detection of *Salmonella* spp. (Santiago-Felipe et al. 2016).

Detection of *Haemonchus contortus*, a biosecurity risk parasite for ruminants, has also successfully been showcased (Melville et al. 2014), with an additional study contrasting LAMP to several other detection methods including (a) McMaster egg counting; (b) counts post staining with peanut agglutinin (PNA); and (c) quantitative polymerase chain reaction (qPCR) (Ljungström et al. 2018). The LAMP assay used in both studies targets the first internal transcribed spacer (ITS-1) with detection in under 1 hour. The initial study that outlined the assays development highlighted the superior 10-fold sensitivity of LAMP when contrasted with conventional PCR, detecting 10 fg and 100 fg of DNA, respectively (Melville et al. 2014). The comparative study indicated that an adapted LAMP assay was second to qPCR but with similar sensitivity results (Ljungström et al. 2018). The authors stated that LAMP is a particularly viable method as it can be applied in resource constrained small diagnostic laboratories, generating sensitive and reliable results in under 1 hour (Ljungström et al. 2018).

The role of LAMP in detecting diseases in tandem to species identification for samples of biosecurity concern could function as an appropriate incursion detection tool at transnational points of entry, particular for live wildlife and domestic animal trade. This has been exemplified with emerging concerns of Foot and Mouth Disease (FMD) incursions globally, and several LAMP assays (Dukes et al. 2006; Bath et al. 2020) developed to address rapid screening. Circumventing resource and time inten-

sive identification methods by utilising LAMP as a point of care diagnostic system could additionally reduce the biosecurity risk posed by potential disease carrier incursions, particularly by reducing the time to outcome and required resources.

Conclusion: LAMP Integration for biosecurity monitoring and surveillance

Recent advances in molecular detection methods have led to the development of simple and cheap devices for the ultrasensitive detection of nucleic acids for clinical diagnosis, food adulteration detection and environmental monitoring (Zhang et al. 2019). This has largely been due to a growing demand for monitoring and detection of nucleic acid biomarkers and the ever-increasing demand for more stringent sensitivity, specificity, and robustness of biomonitoring technologies (Zhang et al. 2019). LAMP methods have emerged as a promising alternative to PCR based systems due to simplicity and point-of-care capabilities (Zhou et al. 2014; Nguyen et al. 2019b; Wan et al. 2019). The ability to conduct LAMP in resource constrained environments where traditional PCR-based technologies may not work, has shown to be highly advantageous in a low resource field-based environment (Raele et al. 2019; Wimbles et al. 2021). Several platforms exploiting isothermal nucleic acid amplification methods have recently become commercially available, widespread, and diverse, including OptiGene (http://www.optigene.co.uk/) Genie systems.

Despite the substantial body of literature, LAMP is yet to receive widespread uptake in research and applied environmental biosecurity monitoring, detections, and enforcement. In the face of globalisation, applying these techniques to DNA-based monitoring in environmental biosecurity contexts is well suited. LAMP as a point of care technology presents great potential for the onsite detection of trace DNA relating to intentional (trafficking) or unintentional (stowaway) transport of live animals, wildlife parts, medicines, and ornamental derivatives. The capacity for LAMP to bridge gaps relating to on-site biosecurity practices, makes it an excellent tool for a range of field-based applications. Furthermore, the low financial, time and resource-based costs render isothermal amplification methods well suited for point of entry detection. Specificity, sensitivity, and robustness comparable to current best practise methods (Francois et al. 2011) allow the integration of these methods into the wildlife forensic science arsenal without compromise (Masters et al. 2019). The ever-increasing interest in LAMP as a point of entry detection method suggests that it may soon function in parallel to PCR, providing widespread molecular diagnostic capacity for biosecurity scenarios.

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SHORT COMMUNICATION

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Genetic relationships among laboratory lines of the egg parasitoid *Trissolcus japonicus* from native and adventive populations

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Abstract

Candidate biological control agents of invasive insect pests are increasingly being found in new geographic regions as a result of unintentional introductions. However, testing the degree of genetic differentiation among adventive and native-range populations of these agents is rarely done. We used reduced-representation sequencing of genomic DNA to investigate the relationships among laboratory lines of *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae), an egg parasitoid and biological control agent of the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera, Pentatomidae). We compared sequences from multiple adventive populations in North America (Canada, USA) and Europe (Switzerland) with populations sourced from part of its native range in China. We found considerably more genetic variation among lines sourced from adventive populations than among those within native populations. In the Pacific Northwest of North America (British Columbia, Canada and Washington State, USA), we found preliminary evidence of three distinct genetic clusters, two of which were highly dissimilar from all

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other lines we genotyped. In contrast, we found that other adventive lines with close geographic proximity (two from Ontario, Canada, three from Switzerland) had limited genetic variation. These findings provide a basis for testing biological differences among lines that will inform their use as biological control agents, and provide evidence to support a hypothesis of several independent introductions of *T. japonicus* in western North America from different source areas.

Keywords
classical biological control, ddRAD, *Halyomorpha halys*, Scelionidae, unintentional biological control

Introduction

There are now numerous documented instances where natural enemies of invasive insects have been discovered following the establishment of their host or prey species, presumably as a result of unintentional introductions (Roy et al. 2011; Weber et al. 2021). Several unintentional introductions are high-profile cases wherein natural enemies were discovered in the insect's invaded range while being evaluated for intentional biological control releases (e.g., Servick 2018; Abram et al. 2020). While molecular techniques have been used to confirm species-level identification of numerous invasive insect pests, determine their invasion history, their geographic origin(s), and possible genetic admixture among them (reviewed in Garnas et al. 2016), these techniques have less often been employed for their natural enemies (but see Lombaert et al. 2014; McCulloch et al. 2022).

In addition to determining invasion histories, molecular techniques could determine relationships among laboratory cultures of adventive natural enemy populations that have been sourced from different regions. When applied to these 'living genetic resources', genetic analyses could identify distinct populations that may differ in biological attributes (e.g., host range, life history, climate tolerance) that affect establishment success and suitability as biological control agents, and could inform future introductions (e.g., to increase genetic diversity of unintentionally introduced populations) or redistributions within new geographic areas that aim to improve biological control outcomes (Abram and Moffat 2018). A range of molecular markers are potentially available for such analyses, however, reduced-representation sequencing (RRS) has not been employed to genotype adventive natural enemies under consideration for biological control programs (Rius et al. 2015; McCartney et al. 2019; Leung et al. 2020). High-resolution genotypes derived from RRS methods could improve our ability to match lab-reared natural enemies with individual populations of invasive species, but these genotypes must first be characterised to permit proper assessment of their safety (potential non-target impacts) and efficacy (host specificity, foraging, and reproductive behaviours) as biocontrol agents.

Trissolcus japonicus (Ashmead) (Hymenoptera, Scelionidae) is an egg parasitoid of the brown marmorated stink bug *Halyomorpha halys* (Stål) (Hemiptera, Pentatomidae) whose presumed native range includes China, southeastern Russia, South Korea, Ja-

pan, and Taiwan (Yonow et al. 2021). In 2014, while *T. japonicus* was being evaluated as a candidate biological control agent, unintentionally introduced populations were detected in the northeastern USA (Talamas et al. 2015a). These findings were followed by detections in the western USA in 2015 (Milnes et al. 2016), Switzerland and eastern Canada in 2017 (Gariepy and Talamas 2019; Stahl et al. 2019), western Canada and Italy in 2018 (Peverieri et al. 2018; Abram et al. 2019), and several other locations throughout Europe and North America thereafter (e.g., Dieckhoff et al. 2021; reviewed in Conti et al. 2021). In the meantime, extensive research has been done on the parasitoid's host range and basic biology (reviewed in Conti et al. 2021; Abram et al. 2022), and intentional introductions of laboratory lines and redistributions of adventive lines are ongoing in Italy and the United States, respectively (Lowenstein et al. 2019; Conti et al. 2021). Stahl et al. (2019) did a preliminary haplotype analysis of the DNA barcode region of the mitochondrial cytochrome *c* oxidase I (COI) gene among populations of *T. japonicus* from Switzerland, Japan, and China, finding five haplotypes among Japanese specimens (one of which was the same as the single haplotype found in Switzerland) and a single base-pair difference between Swiss and Chinese specimens. However, single-marker analyses can be insufficient for detecting species- or population-level genetic structuring (Roe and Sperling 2007; Dupuis et al. 2012; Roe et al. 2017). Thus, in the Pacific Northwest of North America (British Columbia and Washington State), it is unclear whether recently discovered *T. japonicus* are one contiguous population or several distinct introductions. Here, we employed a RRS method, double digest restriction-site associated DNA sequencing (ddRADseq), to: (i) characterise the genetic relationships among laboratory lines established from populations in the native and adventive ranges of this parasitoid; and (ii) begin to evaluate the invasion history of *T. japonicus* in the Pacific Northwest.

Materials and methods

Insect collection and rearing

Between 2017 and 2020, we established 19 laboratory lines of *T. japonicus* in a containment facility certified by the Canadian Food Inspection Agency at Agriculture and Agri-Food Canada's Agassiz Research and Development Centre (Agassiz, British Columbia, Canada). We originally collected progenitors of these lines from wild populations in Switzerland, China, the USA, and Canada between 2009 and 2021 (Fig. 1; Suppl. material 1: table S1). We established 16 iso-female lines by taking a single female from a field-collected parasitized pentatomid egg mass and rearing at least 10 generations of its offspring. We established three additional lines using multiple individuals and reared each as mixed laboratory lines (Suppl. material 1: table S1). For each country of collection, we established two to eight lines. We reared all lines on *H. halys* egg masses following Wong et al. (2021). Elijah Talamas (Florida Department of Agriculture and Consumer Services) or Francesco Tortorici (Department of Agricultural,

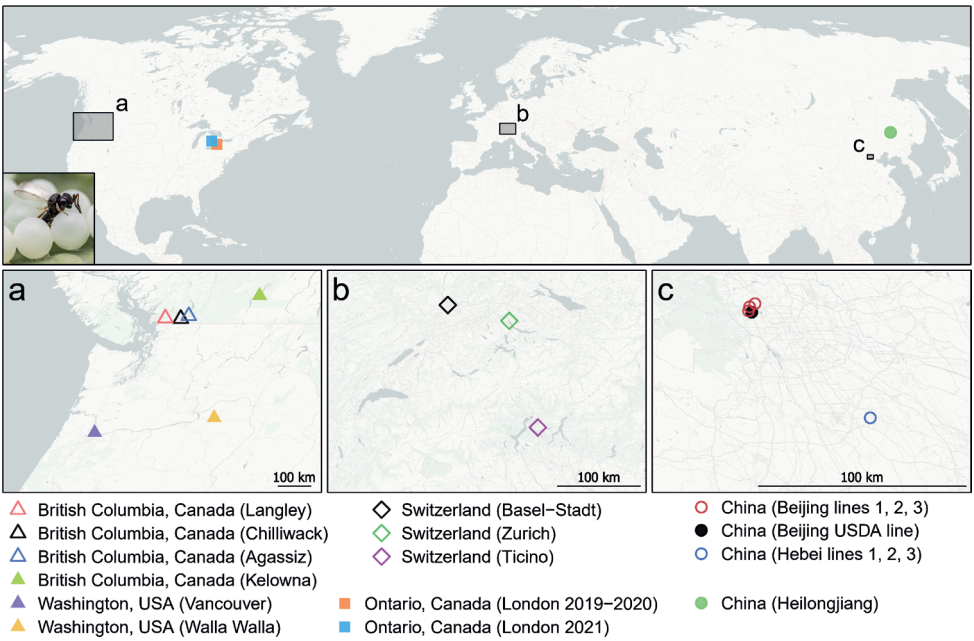

Figure 1. Collection locations for 19 laboratory lines of *Trissolcus japonicus*. Inset maps depict sampling regions **a** the Pacific Northwest of North America **b** Switzerland, and **c** Beijing and Hebei provinces, China. Symbol shape depicts geographic area of collection: diamond = Switzerland, triangle = Pacific Northwest of North America, circle = China, square = Ontario, Canada. Square symbols for the two locations in London, Ontario, Canada have been jittered for visibility.

Forest and Food Sciences, University of Torino) identified vouchers of each line to species-level (Talamas et al. 2015b; Talamas et al. 2017). We deposited representative vouchers in the Florida State Collection of Arthropods; the Canadian National Collection of Insects, Arachnids, and Nematodes; the Summerland Research and Development Centre arthropod collection; and the Royal British Columbia Museum.

DNA extraction and sequencing

We extracted genomic DNA using DNeasy Blood and Tissue DNA kits (QIAGEN, Hilden, Germany) by following the manufacturer's protocol, but we added a bovine ribonuclease A treatment (RNaseA, 4 uL at 100 mg/mL, QIAGEN) to digest RNA. We eluted DNA into 2× 50 uL of 56 °C Buffer AE to increase DNA concentration and yield, then we stored DNA at -20 °C until ddRAD library preparation. *PstI-MspI* library preparation and sequencing were performed by sequencing facility staff as outlined in MacDonald et al. (2020) on an Illumina NextSeq 500 at the University of Alberta (Edmonton, Alberta, Canada). We generated single-end 75 base-pair reads in two separate sequencing runs; we sequenced two samples twice to assess run effects.

Obtaining high-concentration, high-quality DNA from small-bodied organisms is an inherent challenge when preparing DNA libraries (e.g., Andersen et al. 2016; Paspati et al. 2019; Campbell et al. 2020), particularly for hymenopteran parasitoids (Cruaud et al. 2018; Gebiola et al. 2019; Ferguson et al. 2020). However, pooling individuals from female iso-lines has become standard practice in RRS and whole genome analyses of hymenopterans (Leung et al. 2020). We determined that extractions from individual *T. japonicus* did not yield enough DNA for successful ddRADseq (<<200 ng, TDN pers. obs.), and as such, we pooled 10 diploid female *T. japonicus* from individual lines for each DNA extraction (hereafter referred to as 'pools') to meet the minimum required concentration of DNA. Comparison of pooled close relatives may artificially inflate the perceived 'true' genetic distance between wild *T. japonicus* populations (e.g., Rodríguez-Ramilo and Wang 2012), however, our primary objective was to determine the genetic relatedness among laboratory lines. Because Bayesian model-based clustering can be more easily influenced by close relatives than other methods (Waples and Anderson 2017; O'Connell et al. 2019), we employed both Bayesian and non-Bayesian analysis to assess genetic structuring. We prepared a minimum of five pools per line (Table 1), however, we could only prepare four pools from the Ontario, Canada 2021 line due to low specimen availability when sampling. Each pool acted as one unit in analyses.

Table 1. The number of individual pools of 10 female wasps from each *Trissolcus japonicus* laboratory line included in each of the three analyses of population genetic structure. See Suppl. material 1: table S1 for additional information about each *T. japonicus* line.

Laboratory line	Number of pools included in dataset		
	full	geographic	Pacific Northwest
British Columbia, Canada (Langley)	7	–	7
British Columbia, Canada (Chilliwack)	5	–	5
British Columbia, Canada (Agassiz)	7	7	7
British Columbia, Canada (Kelowna)	7	7	7
Washington, USA (Vancouver)	5	4	4
Washington, USA (Walla Walla)	5	–	3
Switzerland (Basel-Stadt)	4	–	–
Switzerland (Zurich)	5	–	–
Switzerland (Ticino)	5	5	–
Ontario, Canada (London, 2019–2020)	4	4	–
Ontario, Canada (London, 2021)	3	–	–
China (Beijing line 1)	5	–	–
China (Beijing line 2)	5	–	–
China (Beijing line 3)	5	5	–
China (Beijing USDA line)	4	–	–
China (Hebei line 1)	5	–	–
China (Hebei line 2)	4	–	–
China (Hebei line 3)	5	–	–
China (Heilongjang)	5	4	–

Bioinformatics

We used Stacks 2 version 2.55 (Rochette et al. 2019) to demultiplex and process raw DNA reads. We removed reads if they 1) contained Phred scores below 20 over 15% of their length, 2) failed the Illumina chastity filter, or 3) had uncalled bases. We used the 'barcode rescue' option to retain reads with one mismatched base in its 8 base adaptor sequence, then we removed all adaptor sequences. Due to some sequencing error in the *PstI* restriction site, we removed an additional 5 bases from the 5' end of each read using Cutadapt version 3.4 (Martin 2011), resulting in final lengths of 62 bases. We used the denovo_map pipeline in Stacks 2 to call single nucleotide polymorphisms (SNPs), specifying one 'population' in the popmap and a minor allele frequency of 0.05. In accordance with Paris et al. (2017), we retained SNPs that were present across at least 80% of the pools. We completed final filtering using VCFtools version 0.1.16 (Danecek et al. 2011), retaining SNPs with a minimum read depth of five and discarding SNPs and pools that had more than 10% missing data, and we used the thin option to keep only one SNP per stack.

We assessed population genetic structuring for three datasets: one containing all 19 laboratory lines ('full dataset'), one with seven lines chosen for proportional representation of potential geographic clusters ('geographic dataset'), and one with all six lines from the Pacific Northwest of North America ('Pacific Northwest dataset') (Table 1). We used principal component analysis (PCA) and Bayesian model-based clustering for assessment. We performed PCA in adegenet version 2.1.5 (Jombart and Ahmed 2011) and we visualised results in ggplot version 3.3.5 (Wickham 2016) using R version 4.1.2 (R Core Team, 2021). We implemented Bayesian model-based clustering in structure version 2.3.4 (Pritchard et al. 2000) using the admixture model. For structure analysis, we randomly sub-selected pools from lines to ensure equal sample size from each (Puechmaille 2016), then ran a burn-in period of 100,000 sweeps followed by 1,000,000 sweeps for each of 10 replications for each potential subpopulation (K) between 1–20 (full dataset) or 1–15 (geographic and Pacific Northwest datasets). We defined each laboratory line in a given dataset as a 'location' prior to better resolving the genetic structure (Porras-Hurtado et al. 2013). We set the alpha prior of the full and geographic datasets to 1/7, and that of the Pacific Northwest dataset to 1/3, reflecting the expected results of K=7 or K=3 (Wang 2017). We assessed statistical support of each K value using LnP(K) (Pritchard et al. 2000) and ΔK (Evanno et al. 2005) in StructureSelector (Li and Liu 2018). Finally, we generated Q-matrices from the 10 replicates of each K for each dataset in CLUMPAK version 1.1 (Kopelman et al. 2015).

Data availability

DNA sequences are available as fastq files in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) as BioProject PRJNA933214.

Results and discussion

Sequencing outcomes and geographic structure

In total, we sequenced 109 pools of *T. japonicus* (1090 individuals) from the 19 laboratory lines, resulting in 109,473,577 raw Illumina reads. In a preliminary PCA, two pools had unexpected behaviour. They may have been contaminated during sample preparation or DNA extraction: neither clustered with any other pool, and both were placed intermediate to other pools extracted from the same line and pools from other lines. We removed both before all subsequent analyses. After our final filtering of raw reads, the full dataset contained 1,889 SNPs across 95 pools with a mean SNP read depth of 64.9×, 11,360,339 filtered reads, and 3.82% total missing data. We used VCFtools to re-filter this dataset before running structure, using the same filtering parameters but sub-selecting near-equal sample sizes for pools from 18 lines in accordance with Puechmaille (2016). We did not include the 2021 line from Ontario, Canada in the full structure dataset because it only had two pools after filtering, leaving a total of 18 lines in the full structure dataset. In 16 of these 18 lines, we subsampled four pools; the other 2 lines (Walla Walla and Beijing-United States Department of Agriculture [USDA]) each had only three pools that passed our filters. Our final full structure dataset had 1,860 SNPs across 70 pools with a mean SNP read depth of 66.0×, 8,469,428 filtered reads, and 1.64% total missing data. In PCA, we identified 6–7 genetic clusters in the full dataset, and we found greatest statistical support for K=9 (LnP(K) method) and K=4 (ΔK method) in the full structure dataset (Fig. 2; Suppl. material 1: figs S1, S2).

In the geographic dataset, we retained 36 pools across the 7 laboratory lines after filtering raw reads. The dataset contained 2,896 SNPs with a mean SNP read depth of 72.9×, 7,498,995 filtered reads, and 2.03% total missing data. We used VCFtools to sub-select four pools per line before running structure, ensuring equal sample size. Our final geographic structure dataset had a mean SNP read depth of 67.4×, 5,386,771 filtered reads, and 2.45% total missing data across 28 pools. We found greatest statistical support for K=11 (LnP(K) method) and K=10 (ΔK method) in this dataset (Fig. 3, Suppl. material 1: figs S3, S4, K=11 structure plot not shown due to similarity with K=10).

In the Pacific Northwest dataset, we retained 33 pools across the 6 laboratory lines after filtering raw reads. This dataset contained 1,976 SNPs with a mean SNP read depth of 78.8×, 5,069,119 filtered reads, and 2.15% total missing data. We used VCFtools to sub-select four pools per line before running structure, ensuring equal sample size; however we could only select three high quality pools from the Walla Walla laboratory line. Our final Pacific Northwest structure dataset had a mean SNP read depth of 71.3×, 3,186,750 filtered reads, and 2.83% total missing data across 23 pools. We found greatest statistical support for K=12 (LnP(K) method) and K=3 (ΔK method) in this dataset (Fig. 4; Suppl. material 1: figs S5, S6).

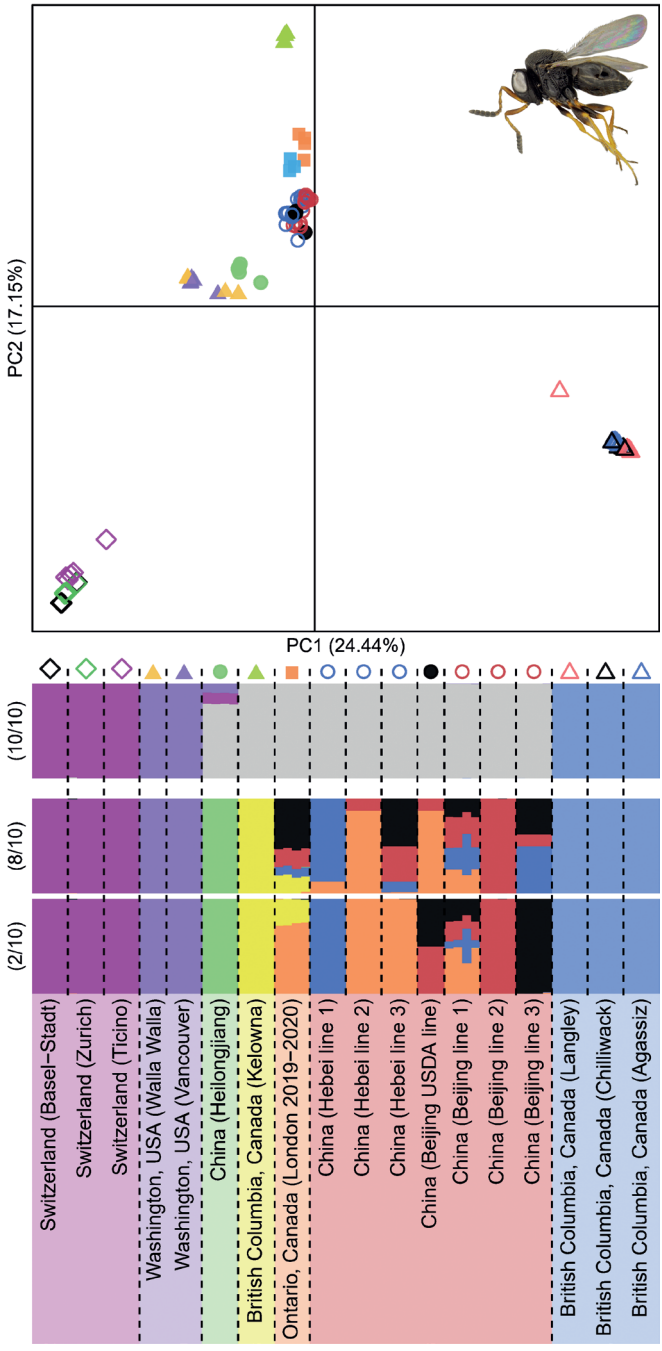

Figure 2. Principal component and structure analyses of SNP data from the full datasets comprising 18–19 *Trissolcus japonicus* laboratory lines. We present structure results with greatest LnP(*K*) and Δ*K* statistical support. Symbol shape depicts geographic area of collection: diamond = Switzerland, triangle = Pacific Northwest of North America, circle = China, square = Ontario, Canada (blue square = London, Ontario, Canada 2021 line). Colours behind laboratory line names correspond with geographic genetic clusters (Fig. 3). We present both modes of *K*=9 across its 10 replicate runs.

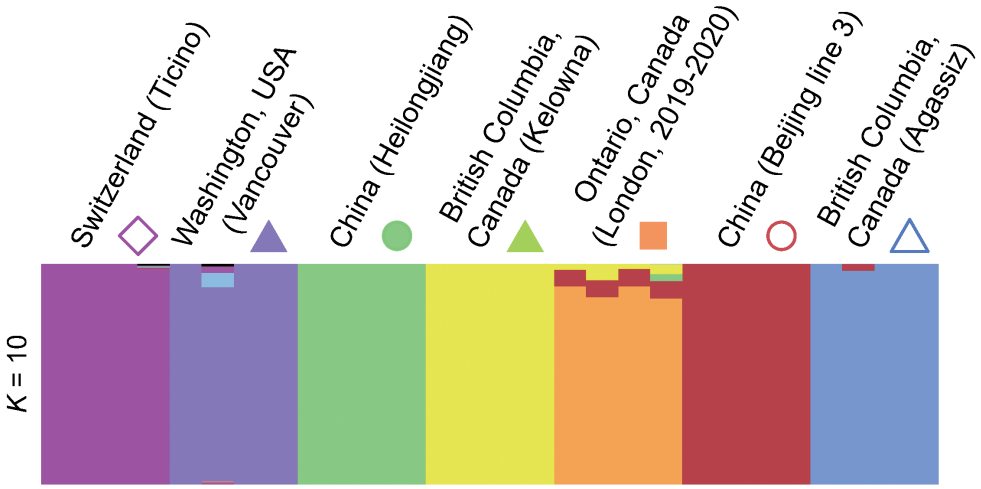

Figure 3. Structure analysis of SNP data from the geographic structure dataset comprising seven *Trissolcus japonicus* laboratory lines. We present structure results with greatest Δ*K* statistical support. Symbol shape depicts geographic area of collection: diamond = Switzerland, triangle = Pacific Northwest of North America, circle = China, square = Ontario, Canada.

Genetic similarities and differences among laboratory lines

Overall, we found *T. japonicus* lines had the greatest genetic similarity when collected in close geographic proximity (Figs 1, 2), but some lines were exceptional (Figs 2, 3). In both PCA and structure analyses, lines collected across Switzerland were more closely related to each other than lines from other countries, as were those from Beijing and Hebei provinces of China (Fig. 2), but lines from Switzerland and Beijing/Hebei were genetically different from one another (Fig. 2). At *K*=4, the Heilongjiang line was included in the same genetic cluster as Beijing and Hebei, however, at *K*=9 it belonged to its own cluster. This could be evidence of isolation by distance across the native range of *T. japonicus* and evidence of one introduction event in Switzerland that did not originate from the areas of China we sampled. In contrast, Stahl et al. (2019) found that the Ticino, Switzerland and Beijing lines had only one base substitution difference in their partial COI sequences (i.e., high apparent similarity) and inferred that the Swiss populations could have originated from the Beijing area. However, our results suggest that neither population-level differentiation nor patterns of invasion history of *T. japonicus* can be evaluated using the mitochondrial genome and thus require a nuclear genome-wide investigation.

Among adventive *T. japonicus* populations in Canada, the lines from London, Ontario and Kelowna, British Columbia were the most similar to populations from China, the parasitoid's native range, suggesting that these two populations may have originated from an area in proximity to our sampled Chinese populations. The two lines from London, Ontario were more closely related to the Beijing and Hebei cluster than was the Heilongjiang line in the full dataset (PCA and structure plots, Fig. 2), but the Ontario, Beijing/Hebei, and Heilongjiang lines each formed their own genetic cluster when proportionally

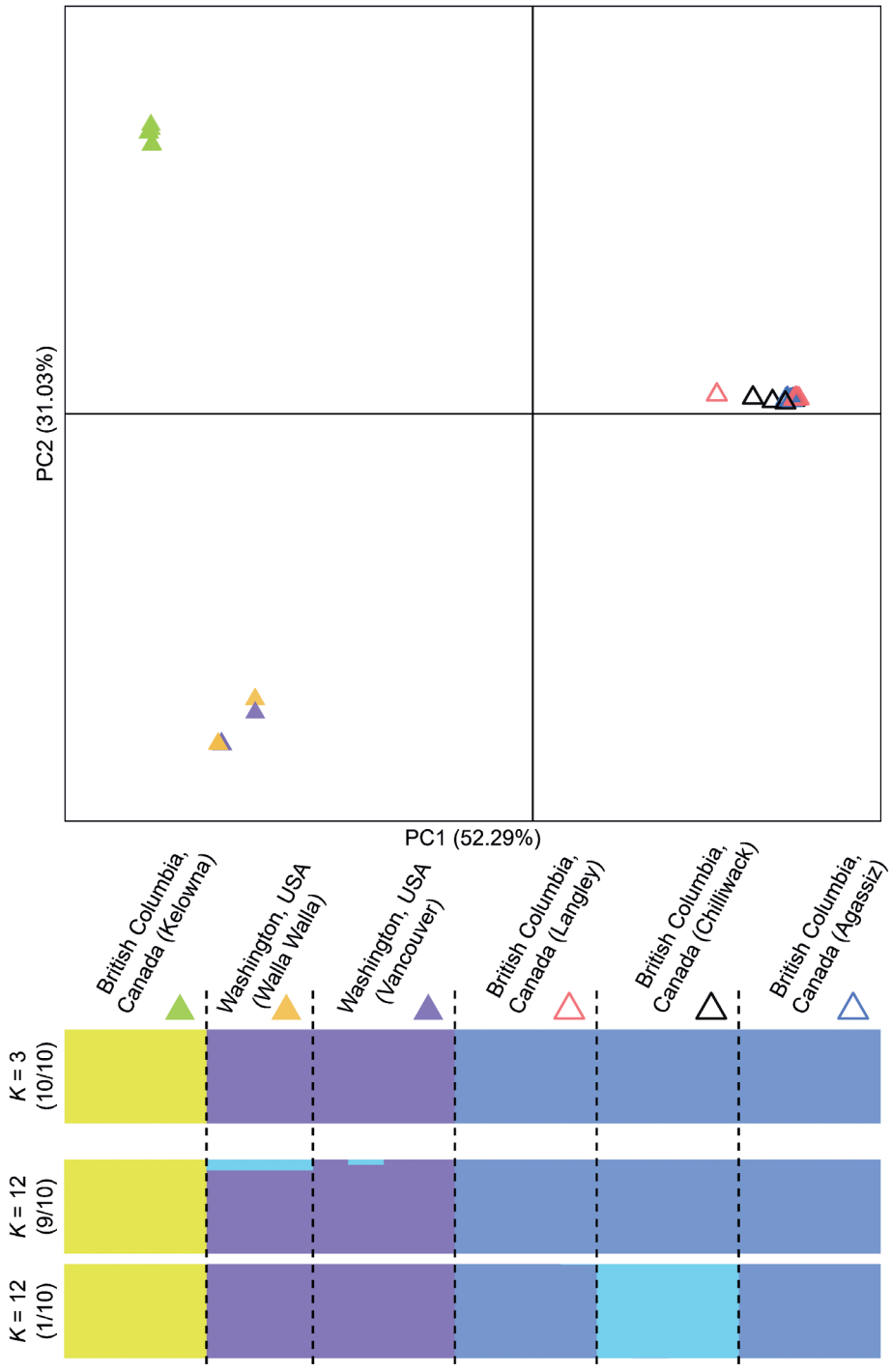

Figure 4. Principal component and structure analyses of SNP data from six *Trissolcus japonicus* laboratory lines collected across the Pacific Northwest of North America. We present the structure results with greatest ΔK and LnP(K) statistical support. We present both modes of K=12 across its 10 replicate runs.

represented in the geographic structure analysis (Fig. 3). It is likely that the seven Beijing/Hebei lines influenced the full dataset by over-representing the central China genotype, as each line is a genetic pseudoreplicate from the same population (see Puechmaille 2016). Nonetheless, this analysis suggests that the Ontario population could have originated from central China. Similarly, the Kelowna line was closely related to the Beijing and Hebei cluster at K=4 but formed its own cluster at K=9, suggesting it too may have been introduced from central China. Further genotyping of *T. japonicus* in its native range will be required to confirm the provenance of adventive populations.

We had expected that westernmost lines from Canada and the USA (Langley, Chilliwack, Agassiz, and Vancouver, WA) would be most closely related, as would those from the interior of BC and WA (Kelowna, BC and Walla Walla, WA). Instead, both lines from Washington State are members of the same cluster despite being separated by more than 350 kilometres, providing good evidence that *T. japonicus* in Walla Walla and Vancouver are either 1) descendants of a single introduction event in Washington or 2) two separate introduction events from the same region (Fig. 4). Likewise, the three lines from western British Columbia (Langley, Chilliwack, and Agassiz) form their own cluster that is genetically dissimilar from those collected in Washington and does not cluster near any other population we sampled. In addition, the newest detection in the Pacific Northwest, Kelowna, forms its own genetic cluster independent of both western BC and Washington State populations, indicating the population in interior British Columbia is likely an independent adventive introduction of unknown provenance and not a dispersion of the other adventive populations of *T. japonicus* in the Pacific Northwest. Strikingly, the amount of genetic variation within the Pacific Northwest lines alone is greater than populations separated by more than 1000 km in the native Chinese range. This suggests that there have been at least three distinct introductions of *T. japonicus* into the Pacific Northwest of North America, and that unintentional introductions of *T. japonicus* from different source areas may be happening with relative frequency. This may be consistent with the introduction history of its host, *H. halys*, in the Pacific Northwest, which appears to have resulted from multiple introduction events as suggested by the occurrence of at least three mitochondrial haplotypes (Abram et al. 2017). However, more extensive analyses using the same genotyping methods would be needed to adequately compare the invasion histories of *H. halys* and *T. japonicus*. In any case, future surveys for genetic and phenotypic variation in *T. japonicus* should not assume that populations in geographically proximal regions are necessarily the result of spread from adjacent regions.

Conclusions

Our study demonstrates that there is relatively strong population genetic structuring between *T. japonicus* laboratory lines collected at relatively small geographic scales, such as the Pacific Northwest of North America. One caveat of these analyses is the relatively low level of biological replication in certain genetic clusters. Nonetheless, clusters with more replicates of independently collected lines (n≥3: Switzerland; Beijing/

Hebei, China; and western British Columbia) did tend to have high genetic similarity relative to the much larger between-cluster variation. Because the geographic limits of these clusters are not yet known, it may be difficult to increase biological replication of the adventive populations. Several regions of the native and adventive ranges of *T. japonicus* are missing from the analyses (e.g., Japan, Italy, Eastern and Central USA), so more work is required to comprehensively describe the worldwide population genetic structure of this species. Secondly, the analyses compared inbred laboratory lines, possibly leading to greater perceived genetic differences between lines than the 'true' wild relationships due to high genetic similarity of each individual in a pool. However, the lines show little evidence of genetic drift towards a common 'lab genotype', and lines that have been in culture for many generations are still genetically similar to more recently established lines from the same genetic cluster, strongly suggesting that these living genetic resources are maintaining their individual integrity and are a close representation of the wild genotypic relationships. To build on this study and clarify the genetic relationships among these laboratory lines, we recommend further research comparing behavioural and life history attributes of each line to inform their use for biological control of *H. halys*. In addition, we suggest that for investigating patterns of invasion history for adventive or invasive species of parasitoids, data from RRS or other genome-wide methods be used, as inferences from single-gene sequencing can over-estimate genetic relatedness among disjunct populations.

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Supplementary material 1

Supplementary information

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RESEARCH ARTICLE

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Effects of invasive *Rosa rugosa* on Baltic coastal dune communities depend on dune age

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Abstract

Rosa rugosa Thunb. (Japanese Rose) is one of the most invasive species in Europe. It spreads spontaneously in coastal areas of western, central and northern Europe, posing a threat to dune habitats, including those indicated in the EU Habitats Directive as particularly valuable. *R. rugosa* has already been reported to displace native plants and alter soil properties. However, little is known about how these effects are mediated by the habitat context or the invader condition (health, ontogenetic stage). This study addressed that gap by examining vegetation and soil in 22 *R. rugosa*-invaded sites, half of which were in yellow dunes and the other half in grey dunes, i.e. two habitats representing the earlier and later stages of dune succession. The study was conducted on the Hel Peninsula (Poland's Baltic coast). *R. rugosa* had a significant impact on dune vegetation, but the impact was strongly dependent on the habitat type. In the yellow dune sites, *R. rugosa* outcompeted most resident plant species, which translated into a strong decline in their total cover and richness. The invasion was almost not accompanied by changes in soil properties, suggesting that it affected the resident vegetation directly (through space takeover and shading). In the grey dunes, *R. rugosa* caused a shift in species composition, from that characteristic of open communities to that typical of forests. In this habitat, a significant increase in the soil organic layer thickness under *R. rugosa* was also observed, which means that both direct and indirect effects of the invasion on the vegetation should be assumed. Finally, a negative relationship was found between the total chlorophyll content in *R. rugosa*

leaves and the parameters of resident plant communities, showing that the invasion effects can vary not only across habitats, but also with the condition of the invader. The results may have practical implications for managing *R. rugosa* invasions in coastal sand dune systems. Since *R. rugosa* accelerates grey dune succession, protecting this habitat may be more urgent and, at the same time, more complicated than protecting dunes at the earlier stages of development.

Keywords
ecological succession, functional trait, grey dune, plant invasion, soil property, species composition, species richness, yellow dune

Introduction

Rosa rugosa Thunb. (Japanese Rose) is a rhizomatous multi-stemmed erect deciduous shrub. The plant is native to northern Japan, the Korean Peninsula, north-east China and the Russian Far East, where it is an essential component of coastal vegetation, such as pioneering communities of sand dunes and rocky and shingle shores, as well as species-rich meadows (Bruun 2005). In China and Japan, *R. rugosa* is considered endemic. In these countries, it is a Red-book species and is legally protected due to a rapid decline in the number of populations caused by anthropogenic pressure (Yang et al. 2009; Nakata et al. 2018). Beyond its native range, *R. rugosa* shows great expansion capacity and has been identified as one of the primary invasive problems in many regions of the world (CABI 2022).

In NW Europe, *R. rugosa* is ranked amongst the seven worst invasive plants (Dassonville et al. 2008). It was originally introduced at the end of the 18th century as a crop and ornamental plant and in the 20th century, it was widely used to stabilise the dunes (Isermann 2008b). It naturalised at the beginning of the 20th century and began to spread spontaneously mainly along sea coasts, becoming widely present between latitudes of 46° and 68° (Bruun 2005). It generally establishes on coastal sand dunes, where it tends to form extensive and impenetrable monodominant thickets that displace local vegetation with high conservation status (Bruun 2005; Isermann 2008b; Kunttu and Kunttu 2017; Perzanowska and Korzeniak 2020) and change the physicochemical properties of the soil and the structure of soil microbial communities (Vanderhoeven et al. 2005; Stefanowicz et al. 2019). Counteracting the *R. rugosa* invasion is a challenge due to the plant's remarkable ability to regenerate after disturbances, such as being buried with sand, uprooted or burned and high resistance to environmental stresses, including drought, frost and salinity (Belcher 1977; Bruun 2005; Kollmann et al. 2011).

Many years of studies have resulted in a good understanding of *R. rugosa* ecology in the areas of invasion. However, there are still gaps in this knowledge. Amongst other things, it is unclear whether and how the influence of *R. rugosa* on local vegetation and soil is modified by the ecological and environmental context. The coastal sand dune systems on which this study focuses are highly heterogeneous as they include dunes

at different stages of maturity – from unstable dunes (yellow dunes) to semi-fixed and fixed dunes (grey and brown dunes) (McLachlan and Defeo 2018). *R. rugosa* enters all types of dunes (Kollmann et al. 2007; Isermann 2008a), which means that it invades plant communities representing all stages of dunes' ecological succession. As these communities obviously differ in species composition and, consequently, in the composition of plant functional traits, they may respond differently to the invasion. There were few attempts to verify this hypothesis and they brought inconclusive results (Isermann 2008b; Thiele et al. 2011). Isermann (2008b) studied the effects of *R. rugosa* on local vegetation depending on its type and showed that the cover and species richness of herbaceous plants (in particular, those typical of coastal grasslands) decreased with the increase of the invader cover and this decrease was stronger in yellow and grey dune communities (i.e. in earlier succession stages) in comparison to brown dune communities. However, the differences in the strength of the revealed relationships were not statistically checked. Additionally, little was said about the nature of invasion-induced changes in the species composition of the studied types of communities. Thiele et al. (2011) investigated the effect of three invasive plants, *Heracleum mantegazzianum*, *Lupinus polyphyllus* and *R. rugosa*, on plant species richness and found that this effect interacted with the type of habitat (or the type of invaded community) for *L. polyphyllus*, but not for the other two species, including *R. rugosa*.

The response of local vegetation to the alien plant invasion may depend on the functional traits of the resident plants, but also on how they interact with the functional traits of the invader (Helsen et al. 2021; Kaushik et al. 2022). Unstable dunes where primary succession takes place are colonised by plant species adapted to hostile environments. Resistance to environmental stresses is important there, while interspecific competition plays a minor role (Olff et al. 1993; Callaway and Walker 1997). *R. rugosa* has both the properties of pioneering species and great ability to compete for space and other resources. Therefore, it is possible that entering the fragile pioneering communities, it displaces the resident plants much easier than in the case of communities of further stages of succession, where interspecific competition is inherently more intense. This conclusion can be drawn from the competitive hierarchy theory, according to which the chance of species co-existence is positively correlated with their functional similarity and, hence, the similarity of their competitive abilities (Fried et al. 2019).

Invasive species affect local vegetation not only directly, but also indirectly by transforming habitat conditions. Those that are capable of creating ecological niches for new species of plants and animals are called invasive ecosystem engineers or transformers (Richardson et al. 2000; Fei et al. 2014). Amongst them are species associated with coastal ecosystems, for example, *Ammophila arenaria*, invasive in North America (Pickart 2021), *Lupinus nootkatensis*, invasive in Iceland (Vetter et al. 2018) and *Senecio inaequidens*, invasive in Europe (Van De Walle et al. 2022). By stabilising the ground surface and producing substantial amounts of organic matter, these invaders accelerate the soil formation process. *R. rugosa* appears to be such a transformer species. First, it can quickly produce a dense cover over a large area (due to the ease of clonal reproduc-

tion) (Kollmann et al. 2009), which effectively limits the mobility of dunes. Second, it is a phanerophyte, i.e. a plant with persistent and relatively high aboveground parts. As such, it can form a canopy that significantly reduces insolation – an important parameter in the coastal environment (Olff et al. 1993) – of the herbaceous layer in which most of the resident dune species occur. Third, it produces considerable amounts of leaf litter (Stefanowicz et al. 2019), which may be a source of limiting nutrients. Putting it all together, invasive *R. rugosa* can be regarded as a transformer leading to acceleration of dune succession. It can also be hypothesised that this role is limited to the early succession stages where sand immobilisation, shading and nutrient supply are potentially of greatest importance for plant colonisation.

Perennial woody plants show the ontogenetic variation that extends over many years. They gradually develop from seedling through the reproductive and ageing stages until they finally begin to die. Along with the successive stages of development, the appearance and vigour of individuals change and, thus, their interaction with other community members (Boege and Marquis 2006; Staska et al. 2014; Lundgren and Des Marais 2020; Qiu et al. 2021). Invasive *R. rugosa* seems to be no exception in this respect. During the fieldwork, we repeatedly observed that some *R. rugosa* patches had a higher ratio of dead to living shoots and, thus, more sparse foliage than others, which appeared to be related to their advanced ontogenetic stage. Perhaps the weakened competitive pressure in such patches opens the way for species previously displaced by the invader to return or for new species to establish.

This study checked whether: 1) the impact of invasive *R. rugosa* on the Baltic coastal dune communities and soils depends on the ecological context, more precisely on the stage of dune succession and whether: 2) the invaded vegetation responds to the ontogenetic variation of the invader. To achieve the study objectives, four types of plots – established in *R. rugosa* patches and adjacent patches of non-invaded local vegetation in both yellow and grey dune sites – were compared in terms of resident plant community parameters (total cover, species richness and composition, functional trait diversity) and soil physicochemical properties. In addition, the surveyed *R. rugosa* patches were described with a number of variables presumably related to the ontogenetic stage or health condition of the invader, including cover, percentage of dead shoots, the content of chlorophyll (Tamary et al. 2019; Zhang et al. 2022) and phenolics in leaves (Borges et al. 2013; Tuominen and Salminen 2017), which were then used to explain coverage, richness and species composition of resident plants.

Methods

Study area and sampling

The study area was the Hel Peninsula (northern Poland). It is a narrow (200–3000 m wide) and very elongated (36 km long) spit separating the Bay of Puck from the Baltic Sea (Fig. 1). The Peninsula is one of the geologically youngest parts of the Polish coast. It formed between 6900 and 1000 years ago from Holocene siliciclastic sediments

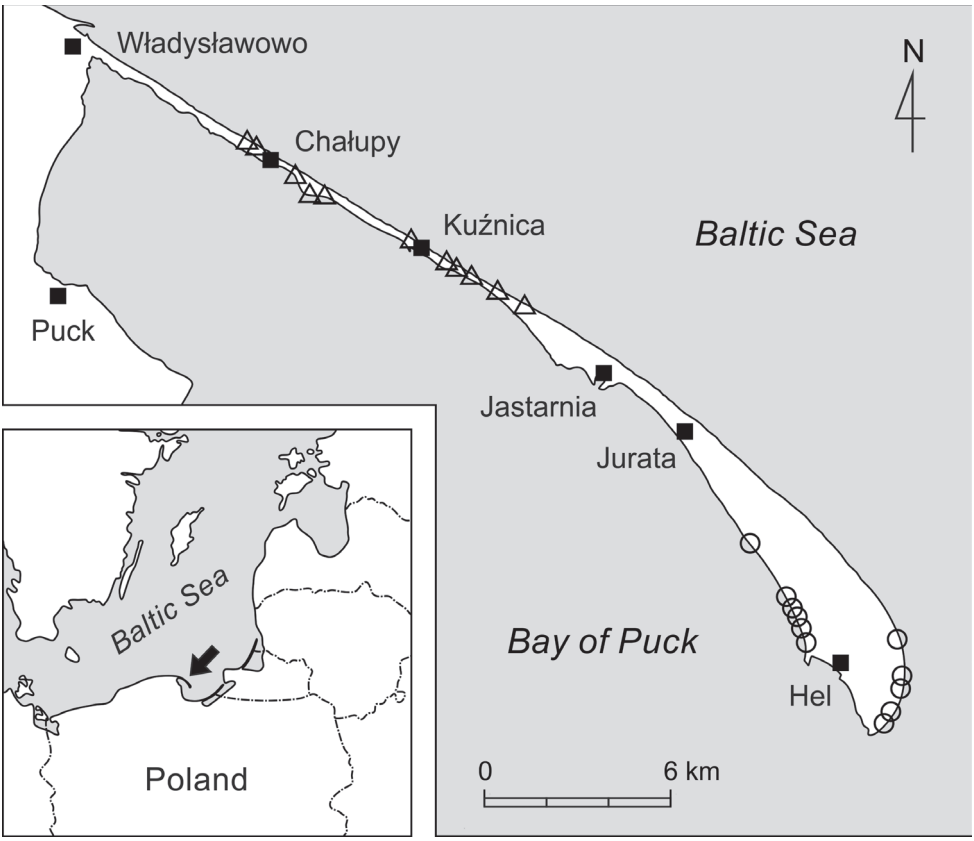

Figure 1. Study area and study sites in four locations: near the villages of Chałupy and Kuźnica and to the west and east of the town of Hel; triangles – yellow dune sites, circles – grey dune sites, black squares – towns and villages.

(mainly sand, less gravel) deposited on the Cretaceous formations by the sea current (Tomczak 1995). The thickness of these sediments reaches 100 m. The relief of the area is nearly level to gently undulating. Its main elements are aeolian dunes, the height of which varies mostly between 3 and 5 m, rarely exceeding 10 m (the maximum height is 22.5 m). Most soils that develop on them are classified as Arenosols (on dunes with unconsolidated or partly consolidated material) and Podzols (on dunes stabilised by forest vegetation) according to the IUSS Working Group WRB (2015). There is no watercourse network, but there are small depressions with periodically wet conditions. The Hel Peninsula lies in the transitional climate zone (shaped by oceanic and continental influences) and has a coastal climate with mild winters and summers. The growing period lasts about 210 days. The average annual temperature is about 9.0 °C. In a year, the rainfall is ca. 750 mm.

The Hel Peninsula is largely covered by the Scots pine and crowberry sub-Atlantic forests of the *Empetro nigri-Pinetum* (Libb. and Siss. 1939 n.n.) Wojt. 1964 association. Between the forest area and the shoreline, there are often zonal strips of loose

shrublands from the *Salicion arenariae* R. Tx. 1952 alliance (the *Rhamno-Prunetea* Rivas Goday and Garb. 1961 class), followed by grassland vegetation typical of the dunes of the sub-Atlantic Central European coastal region. The latter is represented mainly by two communities representing the earlier and later succession stages: 1) tall-grass perennial swards of the *Elymo-Ammophiletum* association Br.-Bl. and De Leeuw 1936 (the *Ammophiletea* Br.-Bl. and R. Tx. 1943 class), hereinafter referred to as the yellow dune community/vegetation and 2) tussock grasslands of the *Helichryso arenarii-Jasionetum litoralis* Libb. 1940 association (the *Koelerio glaucae-Corynephoretea canescentis* Klika in Klika and Novak 1941 class), hereinafter referred to as the grey dune community/vegetation. Both these communities were included in this study as they are most endangered due to the invasion of *R. rugosa*.

The study was conducted in 22 sites established in four different locations: near the villages of Chałupy (n = 5) and Kuźnica (n = 6) and to the west (n = 5) and east (n = 6) of the town of Hel (Fig. 1). The sites were selected so as to include a patch of invader thicket (large and dense enough; Table 1) and a patch of resident grassland vegetation adjacent to the former. One circular sample plot of 4 m^2 was established in each patch, which gives a total of 44 sample plots. The edge-to-edge distance between plots in a pair, i.e. between the invasion plot and the resident vegetation plot (control), was kept as small as possible; it ranged from two to five metres across the sites. Another criterion for selecting the sites was that they should evenly represent the two dominant types of grassland communities. Thus, 11 sites were established in the area of the occurrence of yellow dune vegetation (Chałupy and Kuźnica locations) and 11 sites were established where grey dune vegetation prevailed (Hel locations). Plant communities were identified in the field using a guide by Matuszkiewicz (2013) based on patches of vegetation not influenced by *R. rugosa*.

Botanical data and soil samples were collected at the end of May 2018. The May date was chosen because it combined the sufficient advancement of the growing season and the relatively low level of anthropogenic disturbance (eutrophication, trampling) related to tourist traffic. At each plot, all vascular plant species were identified and their cover-abundances were estimated using the seven-grade Braun-Blanquet scale: r (< 5%, one small individual), + (< 5%, one to three individuals), 1 (< 5%, several individuals), 2 (5–25%); 3 (25–50%), 4 (50–75%) and 5 (75–100%). Species nomenclature followed Mirek et al. (2002). From each plot, three samples of mineral soil were taken

Table 1. Characteristics of *Rosa rugosa* thickets (means ± standard deviations) for the yellow and grey dune sites. Note that total phenolics content was expressed as Tannic Acid Equivalent (TAE). Fv/Fm – the maximum photochemical efficiency of photosystem II (for explanation, see the text).

Variable	Yellow dune sites, n = 11	Grey dune sites, n = 11
Area (m^2)	204 ± 142	113 ± 129
Coverage (%)	92 ± 8	89 ± 6
Annual shoots (%)	6.4 ± 7.8	9.5 ± 5.2
Dead shoots (%)	6.8 ± 7.8	18.2 ± 13.5
Total chlorophyll (mg g^{-1})	1.54 ± 0.23	1.52 ± 0.15
Fv/Fm	0.753 ± 0.061	0.711 ± 0.098
Total phenolics (mg TAE g^{-1})	51.0 ± 2.0	51.2 ± 2.6

(after removal of the organic layer, if present) from a depth of 0–10 cm and bulked to obtain one composite sample. At the soil sampling spots, the thickness of the organic layer was measured and averaged over the plot. Additional work was done in the invasion plots. The percentage of annual and dead *R. rugosa* shoots was estimated. Additionally, fragments of vivid shoots with leaves were randomly collected from *R. rugosa* (from three individuals per plot) for the chlorophyll fluorescence analysis. Each fragment was wrapped with a moistened paper towel, placed in a separate plastic container and transported to the laboratory on the same day. In October 2018, senescing *R. rugosa* leaves were sampled (from three individuals per plot) for analysis of phenolics content; they were kept frozen at –20 °C until analysis.

Chlorophyll content and fluorescence analysis

The leaf content of chlorophyll was analysed according to Barnes et al. (1992). Fresh material of the *R. rugosa* leaves was extracted in dimethyl sulphoxide (SIGMA-Aldrich, St. Louis, MO, USA) at 65 °C for 12 h. The chlorophyll was measured spectrophotometrically at 648 and 665 nm with a CECIL spectrophotometer (Cambrige, United Kingdom). Its total content was calculated using the formula:

$$[(7.49 \times A_{665} + 20.34 \times A_{648}) \times V)] / (1000 \times W),$$

where A is absorbance of wavelength (nm), V is the volume of the extract (ml) and W is the weight of the sample (g).

The chlorophyll *a* fluorescence was determined with a fluorimeter (Hansatech, United Kingdom). The second leaves were acclimatised to the dark for 30 minutes using clips. After this time, leaves were exposed to excitation light (1000 μmol m^{-2} s^{-1}) for 1 s (Lichtenthaler et al. 2004). Amongst the measured parameters were: F0 – zero fluorescence, Fm – maximal fluorescence and Fv/Fm – the maximum photochemical efficiency of photosystem II (PSII), where Fv = Fm – F0.

Analysis of soil physicochemical properties

Depending on the analysed properties, the soil samples were dried either at room temperature (pH, electrical conductivity, content of $N\text{-}NH_4$, $N\text{-}NO_3$ and $P\text{-}PO_4$) or at 105 °C (organic C content and total content of N, P, Na and Ca) overnight and then sieved to 1 mm. The pH (ISO 1994) and electrical conductivity (PN-ISO 1997) were measured after dilution of soil in distilled water (1:5, w:v) using a Hach HQ40D multi-meter. For $N\text{-}NH_4$, $N\text{-}NO_3$ and $P\text{-}PO_4$ analysis, soil samples were shaken in water (1:10, w:v) for 1 h using a 358S shaker (Elan) and then passed through cellulose acetate syringe filters with a pore size of 0.45 μm (Huang and Schoenau 1998; modified). The content of $N\text{-}NH_4$ in the extracts was determined using an ion chromatograph Dionex DX-100, while that of $N\text{-}NO_3$ and $P\text{-}PO_4$ was determined using Dionex ICS-1100. The contents of elements were determined for soil samples ground

by a vibratory mill (Analysette 3 Spartan Pulverisette 0, Fritsch). Organic C content was measured with a Leco RC-612 (ISO 1995). Total N content was analysed by the Kjeldahl method, which included soil mineralisation in H_2SO_4 with Kjeltabs (K_2SO_4 + $CuSO_4 \cdot 5H_2O$; Foss Tecator Digestor Auto 20) and distillation using a Foss Tecator Kjeltec 2300 Analyser Unit (AN 300 Ver. 4.0). Prior to the determination of the total content of Ca, Na and P, soil was mineralised in suprapur $HClO_4$ (Foss Tecator Digestor Auto 40). The metals were analysed with a flame atomic absorption spectrometer (AA280FS, Varian), while P was analysed with a colorimeter (DR 3800, Hach Lange) using the vanadate-molybdate method (Nowosielski 1974).

Data handling and analysis

Each plant species recorded in this study was characterised using several categorical functional traits. They were: functional group identity (forbs, graminoids, legumes, woody plants), C-S-R life strategy, life form, seed dispersal type and pollination type (Klotz et al. 2002; Kleyer et al. 2008). For each plot, the number of species representing a given function (trait category), the total number of species (species richness) and the total plant coverage were calculated. To determine the latter variable, species cover-abundance values expressed using the seven-grade Braun-Blanquet scale were converted to equivalent percentage cover values, 1%, 2%, 3%, 13%, 38%, 63% and 88% (Tichý et al. 2020) and then summed up. Summation results often exceeded 100% due to the fact that the vegetation has a multi-layered structure. Only the resident plant species were included in the above calculations, i.e. all except *R. rugosa*. The number of endangered species (protected and/or rare), as defined by the Regulation of the Minister of the Environment of 9 October 2014 on the protection of plant species (Ministry of Environment of the Republic of Poland 2014) and the Polish Red List (Kaźmierczakowa et al. 2016), was also calculated.

Prior to statistical analysis, the data were transformed to reduce variability and approximate normality: total coverage and species richness of resident plants, *R. rugosa* thicket characteristics and soil properties were log-transformed and then normalised, while resident plant species cover-abundances and functional traits (i.e. species numbers in functional trait categories) were square-root transformed (Anderson et al. 2008). Variables carrying little information were not taken into account in multivariate analyses; these were species occurring as singletons and functional traits rarely found in the studied communities (represented by less than two species and/or absent in ≥ 75% of the study plots).

The effects of plot type (invasion vs. control plots), site type (yellow vs. grey dune sites) and the interaction of these factors on the total cover and species richness of resident plants and the number of protected plant species were determined using linear mixed-effects (LME) models. Two random factors were included in the models: site, within which plots were nested and location, within which sites were nested. The models were fitted using the "nlme" R package (Pinheiro et al. 2017) and their assumptions were checked with diagnostic tools ("plot.lme" function) provided with the package. The datasets on the plant functional traits and soil properties contained many

Table 2. Soil properties (means ± standard deviations) for the control (C) and invasion (I) plots within the yellow and grey dune sites and the effects of site type, plot type and their interaction on these properties, as shown by *F*-values derived from the LME analysis. Significant effects are marked with asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Variable	Yellow dune sites		Grey dune sites		Effects		
	C plots, n=11	I plots, n=11	C plots, n=11	I plots, n=11	Site type	Plot type	Interaction
Organic layer thickness (cm)	1.3 ± 1.2	1.7 ± 1.1	1.2 ± 0.8	2.6 ± 1.3	0.1	21.2***	7.1*
pH	6.2 ± 0.7	6.2 ± 0.6	5.5 ± 0.3	6.0 ± 0.4	1.4	11.7**	12.4**
Electrical conductivity (μS cm^{-1})	15.3 ± 4.3	13.6 ± 2.8	11.8 ± 2.8	13.4 ± 4.1	0.6	0.0	4.2
Organic C (%)	0.33 ± 0.34	0.44 ± 0.42	0.14 ± 0.14	0.20 ± 0.13	0.7	3.8	0.0
Total N (%)	0.018 ± 0.016	0.032 ± 0.032	0.009 ± 0.007	0.014 ± 0.008	0.7	4.5*	0.4
C/N	20.3 ± 28.5	22.2 ± 27.4	23.9 ± 26.5	14.0 ± 4.4	0.0	0.2	1.0
Total P (mg kg^{-1})	94 ± 45	103 ± 40	89 ± 45	57 ± 25	1.0	1.4	4.5*
Total Na (mg kg^{-1})	8.3 ± 2.6	8.7 ± 2.7	5.5 ± 1.2	4.5 ± 0.9	22.9*	1.2	3.8
Total Ca (mg kg^{-1})	339 ± 171	353 ± 146	261 ± 88	266 ± 162	1.4	0.0	0.2
$N\text{-}NH_4$ (mg kg^{-1})	0.66 ± 0.34	0.77 ± 0.42	0.65 ± 0.39	0.56 ± 0.27	3.8	0.1	1.5
$N\text{-}NO_3$ (mg kg^{-1})	0.29 ± 0.29	0.32 ± 0.39	0.10 ± 0.06	0.10 ± 0.03	0.5	0.1	0.5
$P\text{-}PO_4$ (mg kg^{-1})	1.16 ± 0.69	1.67 ± 0.85	0.91 ± 0.44	1.47 ± 0.53	0.1	25.0***	0.2

variables, to some extent correlated with each other and, therefore, they were analysed using a multivariate approach – permutational multivariate analysis of variance (PERMANOVA). The PERMANOVA models had the same error term structure as the LME models and were based on Euclidean distances. Data on species abundances were analysed in the same way, except that Bray-Curtis distances were used. For the species data, in addition to PERMANOVA, similarity percentage (SIMPER) analysis (Anderson et al. 2008) with a 50% contribution cut-off point (Clarke 1993) was performed on the Bray-Curtis distance matrix to identify species that contributed most to the differences between invasion and control plots. To visualise the results of PERMANOVAs, principal coordinates analysis (PCoA) ordinations were generated, wherein plots were symbol-coded according to plot and/or vegetation type. Each PCoA was performed on exactly the same data as the corresponding PERMANOVA.

The variability in the total coverage of the resident plants, as well as their species richness and composition across the invasion plots, was explained using distance-based linear models (DistLM) (Anderson et al. 2008). The explanatory variables were preselected from Tables 1, 2; they were: four parameters of *R. rugosa* thickets (thicket area, percentage of annual shoots, total chlorophyll content and total phenolics content) and seven soil properties (the contents of organic C, total Na, total Ca, $N\text{-}NH_4$, $N\text{-}NO_3$ and $P\text{-}PO_4$ and the C/N ratio). Pre-selection was made on the basis of the variance inflation factor (VIF) provided with the "car" R package (Fox and Weisberg 2011) and its aim was to reduce the multi-collinearity in the explanatory dataset; VIF for the variables used in the analysis was < 4. Forward selection procedure and the AICc criterion were used to obtain the best models explaining the parameters of resident plant communities. For total coverage and species richness, DistLM was based on Euclidean distances, while, for species composition, it was based on Bray-Curtis distances. To visualise the results of DistLM for species composition, distance-based redundancy analysis (dbRDA) was used.

PERMANOVA, PCoA, DistLM and dbRDA routines were executed using PRIMER 7 with the PERMANOVA+ package (Anderson et al. 2008). Other analyses were carried out in R 3.3.3 (R Core Team 2020).

Results

A total of 55 species of resident vascular plants (i.e. other than *R. rugosa*) were found in this study, including four endangered species: *Agrostis vinealis*, *Epipactis atrorubens*, *Festuca polesica* and *Lathyrus japonicus* ssp. *maritimus*. Amongst them, there were 11 ubiquitous species, i.e. occurring in both types of sites, yellow and grey dune sites and in both types of plots, invasion and control plots (Suppl. material 1: fig. S1). There were almost twice as many species composing the yellow dune communities (44 species) as those composing the grey dune communities (25 species). The difference was more pronounced in the case of unique species, i.e. exclusive to a given type of community; there were 30 species exclusive to the yellow dune community and only 11 species exclusive to the grey dune community. The control plots did not differ much from the invasion plots in the total number of species recorded therein; the former harboured 42 species, including 16 exclusive ones, while the latter harboured 39 species, including 13 exclusive ones (for shared and exclusive species lists, see Suppl. material 1: table S1).

The number of resident plant species per plot (species richness) ranged from 2 to 13, averaging 6.9 (median = 6.5) and their total coverage varied between 8% and 209%, averaging 61.9% (median = 54.5%). Both parameters differed statistically significantly between the invasion and control plots, but only within the yellow dune sites, as evidenced by the interaction effect (site type × plot type; Table 3) and post-hoc comparisons (Fig. 2A, B). The yellow dune vegetation of the control plots was characterised by about four times higher coverage (117%) and one and half times higher species richness (9.4 species per plot) than the yellow dune vegetation of the invasion plots (30% and 6.7 species per plot). Within the grey dune sites, the vegetation of the two types of plots was similar both in terms of total coverage (49% and 51% in the control and invasion plots, respectively) and species richness (5.3 and 6.2 species per plot in the control and invasion plots, respectively).

The species composition of resident plants was also influenced by the site type × plot type interaction (Table 3). Unlike in the case of univariate parameters described above, the difference between the control and invasion plots was pronounced within the grey dune sites ($t = 3.8$, $p < 0.001$), while, within the yellow dune sites, it was on the verge of statistical significance ($t = 1.5$, $p = 0.056$). PCoA diagrams (Fig. 3A, B) and SIMPER analysis (Suppl. material 1: table S2) showed that the compositional shift from the control towards invasion plots within the grey dune sites was mainly due to a considerable decrease in the abundance (expressed in both cover and frequency; cf. Suppl. material 1: table S1) of dominant species of graminoids, *Corynephorus canescens* and *Ammophila arenaria* and an increase in the abundance of *Festuca villosa*. Similar results were obtained in the analysis of functional traits; their composition was shaped by the interaction of factors (Table 3) and a more pronounced compositional

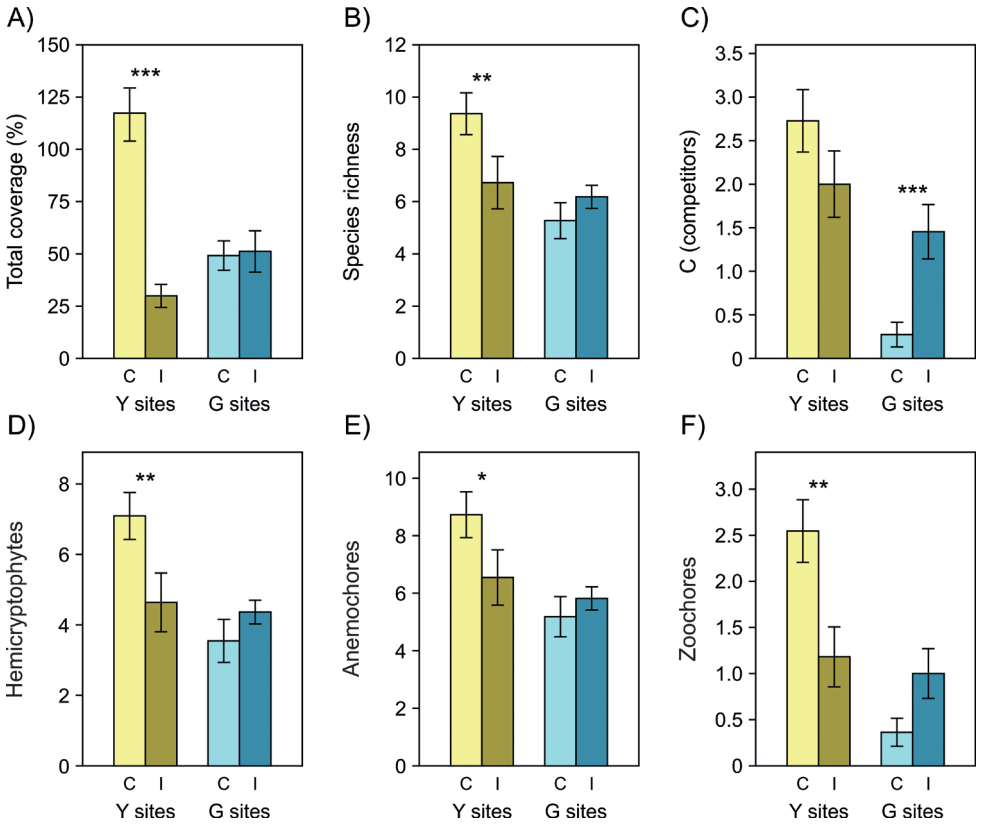

Figure 2. Means and standard errors of selected plant community parameters calculated for the C (control) and I (invasion) plots within the Y (yellow dune) and G (grey dune) sites. As a significant site type × plot type interaction effect was found for all variables (Table 3; Suppl. material 1: table S3), the differences between plot types were examined separately for the two types of sites (using Tukey's test) and marked with asterisks (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

Table 3. Effects of site type, plot type and their interaction on dune vegetation and soil, as shown by *F* and *p* values derived from the LME analysis (for univariate data; rows indicated by [L]) and pseudo-*F* and permutation *p* values derived from PERMANOVA (for multivariate data; rows indicated by [P]). Statistically significant effects are in bold.

	Site type		Plot type		Interaction	
	F	*p*	*F*	*p*	*F*	*p*
Total coverage [L]	1.1	0.4044	**29.3**	**< 0.0001**	**25.3**	**< 0.0001**
Species richness [L]	4.7	0.1633	1.5	0.2366	**8.7**	**0.0080**
Species composition [P]	2.9	0.3299	**6.5**	**0.0007**	**5.1**	**0.0011**
Functional traits [P]	3.2	0.3333	1.5	0.1852	**5.4**	**0.0016**
Soil properties [P]	1.1	0.6669	**3.2**	**0.0052**	**2.2**	**0.0399**

shift between plot types was observed within the grey dune sites (grey dune: $t = 1.9$, $p = 0.0160$; yellow dune: $t = 1.8$, $p = 0.0445$). PCoA diagrams (Fig. 3C, D) and univariate tests (Suppl. material 1: table S3; Fig. 2C–F) suggested that the differences in the

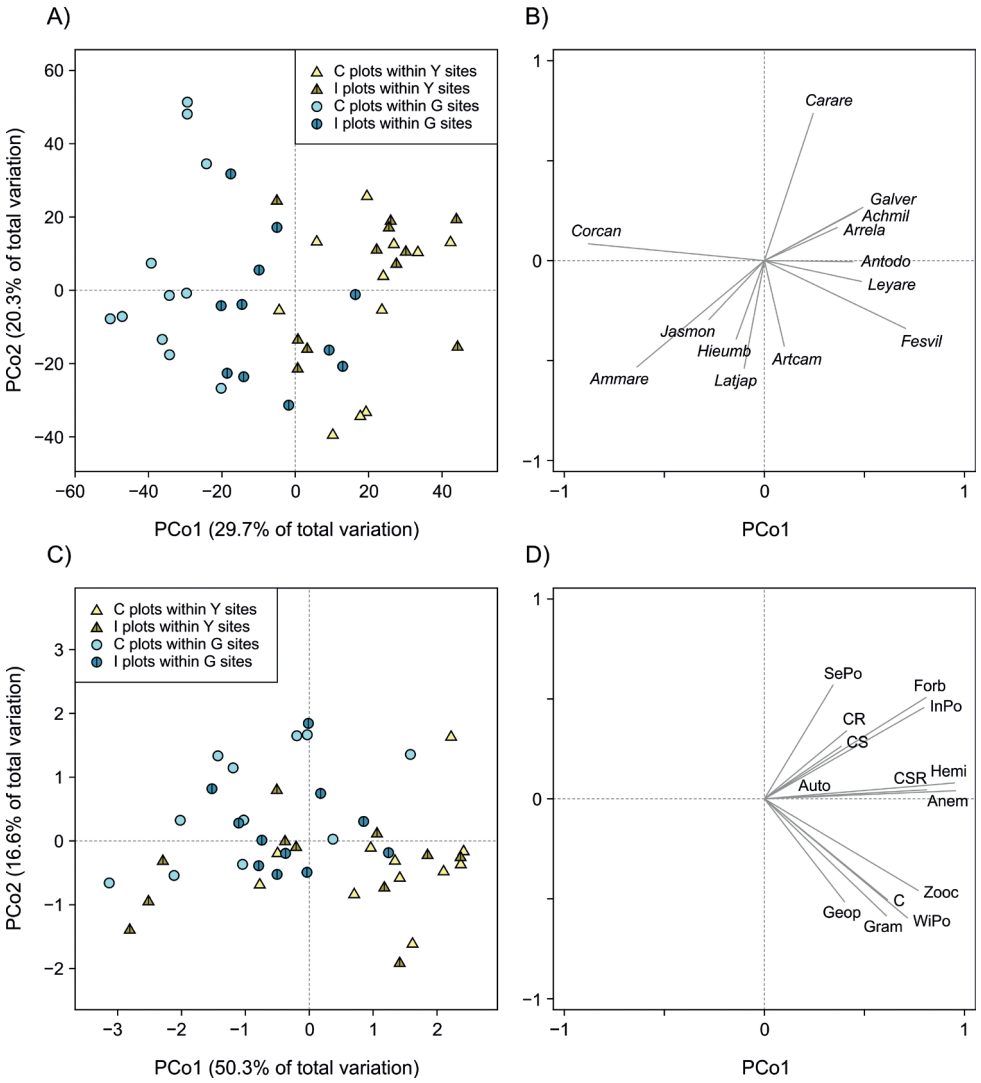

Figure 3. The results of principal coordinates analysis (PCoA) for resident plant species (**A, B**) and functional trait (**C, D**) data. PCoAs were based on Bray-Curtis and Euclidean distances, respectively. The left diagrams (**A, C**) show the position of C (control) and I (invasion) plots established within the Y (yellow dune) and G (grey dune) sites in the ordination space. The right diagrams (**B, D**) show the projection of species (**B**) and functional traits (**D**) on to the ordination space; for clarity, only species that correlated best ($r > 0.4$) with the PCoA axes were displayed. Explanation of acronyms of species names: *Achmil* – *Achillea millefolium*, *Ammare* – *Ammophila arenaria*, *Antodo* – *Anthoxanthum odoratum*, *Arrela* – *Arrhenatherum elatius*, *Artcam* – *Artemisia campestris* ssp. *sericea*, *Carare* – *Carex arenaria*, *Corcan* – *Corynephorus canescens*, *Fesvil* – *Festuca villosa*, *Galver* – *Galium verum*, *Hieumb* – *Hieracium umbellatum* var. *dunense*, *Jasmon* – *Jasione montana* var. *litoralis*, *Latjap* – *Lathyrus japonicus* ssp. *maritimus*, *Leyare* – *Leymus arenarius*. Explanation of functional trait acronyms: Forb – forbs, Gram – graminoids, C – competitors, CR – competitive ruderals, CS – stress-tolerant competitors, CSR – mixed strategists, Geop – geophytes, Hemi – hemicryptophytes, InPo – insect-pollinated, SePo – self-pollinated, WiPo – wind-pollinated, Anem – anemochores, Auto – autochores, Zooc – zoochores.

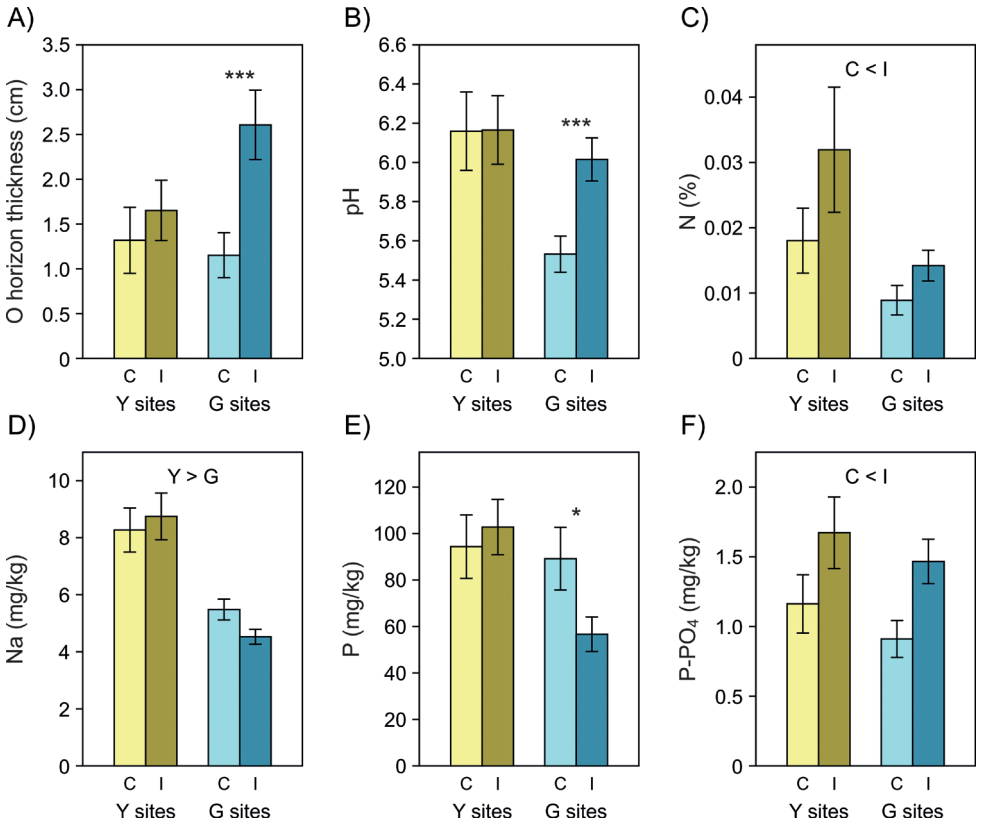

Figure 4. Means and standard errors of selected soil properties calculated for the C (control) and I (invasion) plots within the Y (yellow dune) and G (grey dune) sites. Where a significant site type × plot type interaction was found (see Table 2), the differences between plot types were examined separately for the two types of sites (using Tukey's test) and marked with asterisks (*** $p < 0.001$, * $p < 0.05$). In the remaining cases, inequality signs were used to indicate significant main effects.

number of competitors, hemicryptophytes, insect-pollinated plants, anemochores and zoochores between plot types contributed the most to the interaction effect. Within the grey dune sites, these groups of species were more numerous in the invasion plots than in the control plots, while, within the yellow dune sites, the opposite was true.

According to PERMANOVA, the two types of plots differ in soil properties and these differences depend on the type of site (as evidenced by the site type × plot type interaction; Table 3); they are statistically significant within the grey dune sites ($t = 2.0$, $p = 0.010$) and insignificant within the yellow dune sites ($t = 1.1$, $p = 0.316$). Univariate tests (Table 2) show that the following variables may have contributed to this result: 1) organic layer thickness and pH, which, within the grey dune sites, were higher in the invasion plots than in the control plots (Fig. 4A, B), 2) total N and P-PO_4, which had a similar pattern, but present in both types of sites (Fig. 4C, F) and 3) total P, which within the grey dune sites was higher in the control plots than in the invasion plots (Fig. 4E).

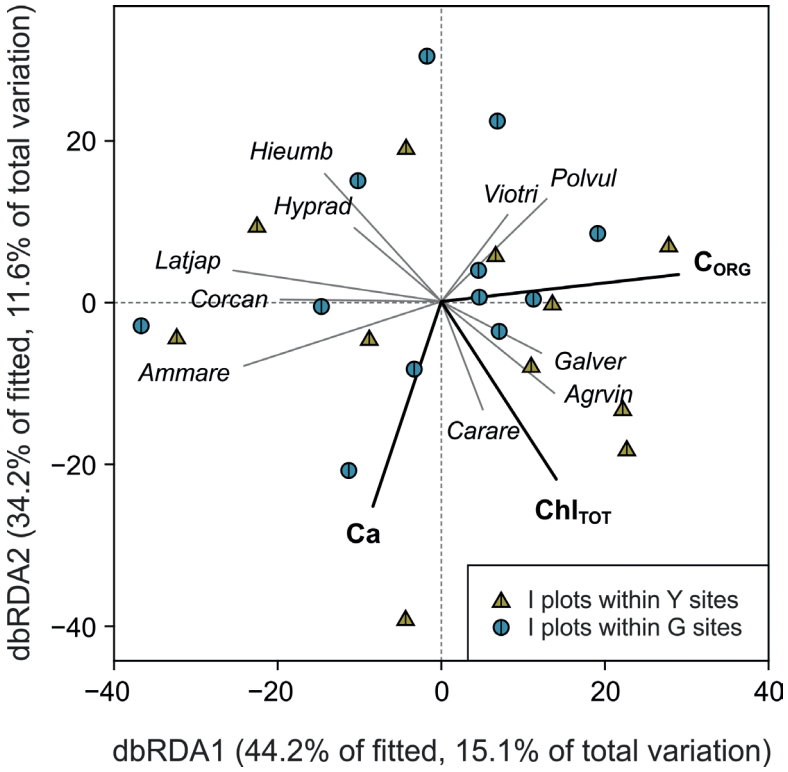

Figure 5. The results of distance-based redundancy analysis (dbRDA) showing the relationship between forward-selected plot characteristics – Ca (soil total Ca), C_{ORG} (soil organic C) and Chl_{TOT} (*Rosa rugosa* leaf total chlorophyll) – and resident plant species occurrence in the invasion plots. The analysis was based on Bray-Curtis distances. Explanation of acronyms of species names: *Agrvin* – *Agrostis vinealis*, *Ammare* – *Ammophila arenaria*, *Carare* – *Carex arenaria*, *Corcan* – *Corynephorus canescens*, *Galver* – *Galium verum*, *Hieumb* – *Hieracium umbellatum* var. *dunense*, *Hyprad* – *Hypochoeris radicata*, *Latjap* – *Lathyrus japonicus* ssp. *maritimus*, *Polvul* – *Polypodium vulgare*, *Viotri* – *Viola tricolor* ssp. *curtisii*

Out of 11 pre-selected habitat variables (four parameters of *R. rugosa* thickets and seven soil physicochemical properties), the DistLM analysis selected three that significantly explained the species composition of resident plants in invaded plots; they were total chlorophyll content in *R. rugosa* leaves (pseudo-F = 2.7, p = 0.012) and the soil contents of organic C (pseudo-F = 3.2, p = 0.001) and total Ca (pseudo-F = 2.7, p = 0.007). The dbRDA diagram (Fig. 5) shows that most species were negatively related to these variables. Total *R. rugosa* leaf chlorophyll was an important explanatory variable in models for total coverage (pseudo-F = 6.4, p = 0.018) and species richness (pseudo-F = 11.7, p = 0.002) of resident plants; it was negatively related to both dependent variables. Other explanatory variables included in these models were total Ca and $N\text{-}NH_4$; the former was positively related to total coverage (pseudo-F = 4.8, p = 0.044) and the latter to species richness (pseudo-F = 5.7, p = 0.030).

Discussion

Non-invaded open dune vegetation

The coastal sand dune system includes dunes at various stages of development, from young, just forming, to mature, fixed dunes (McLachlan and Defeo 2018). They create a spatial gradient of habitat conditions running approximately inland from the shoreline. Ideally, such a gradient extends widely and covers a set of plant community types representing subsequent stages of dune ecological succession – from pioneering communities to forest communities (Peyrat and Fichtner 2011; Doody 2013; McLachlan and Defeo 2018). On the Hel Peninsula, where our study was conducted, the above gradient is relatively short. This is due to the narrowness of the land (most of the Peninsula is less than 1 km wide) and its anthropogenic transformations – the inner dunes were stabilised mainly by Scots pine afforestations, while those closest to the sea shore, especially near the beach entrances, were planted with species of grasses and shrubs, including *R. rugosa* (Mankowski 1906; Łabuz 2013). As a result, the strip of spontaneous open dune vegetation is strongly narrowed and usually dominated at a given place by one of its types: in the western part of the Peninsula, i.e. in an area with high geomorphological dynamics, yellow dune vegetation prevails, while in the eastern part of the peninsula, i.e. in an area with more stabilised landforms, grey dune vegetation is more common (note that this situation is reflected in the distribution of study sites; Fig. 1).

Despite the significant geographical separation between the two types of sites, the studied communities had some common features. They shared several plant species that are important components of dune grassland ecosystems, such as *Ammophila arenaria*, *Artemisia campestris* ssp. *sericea*, *Carex arenaria*, *Corynephorus canescens*, *Festuca villosa*, *Hieracium umbellatum* var. *dunense*, *Lathyrus japonicus* ssp. *maritimus* and *Leymus arenarius*. Moreover, they developed on substrates with very similar physicochemical properties. The yellow dune soils differed from the grey dune soils only in pH and Na content. These parameters were higher for the former, which is a typical result of more intense coastal deposition (sea spray, fresh sand, remains of marine organisms, for example, shells) in places closer to the sea (Hundt 1985; Isermann 2005; Ogura and Yura 2008; Rajaniemi and Allison 2009; McLachlan and Defeo 2018).

Regarding the dissimilarities between the studied communities, they were more obvious than the similarities. Firstly, there were many species present in yellow dune communities, but absent in grey dune communities. This translated into a significantly higher species richness and total plant cover in the former. Secondly, shared species usually showed strong affinities for one type of community, as evidenced by records of their frequencies and abundances (Suppl. material 1: table S1). For example, *Hieracium umbellatum* var. *dunense* was common in both types of communities, but only in yellow dune communities did it reach high cover values; in contrast, *Corynephorus canescens* was always present and abundant on grey dunes, while on yellow dunes, it was observed only twice. The clear separation of points representing the two types of

non-invaded (control) plots in the PCoA diagrams (Fig. 3A, C) confirms the significance of the between-community qualitative differences.

When selecting the yellow and grey dune sites for this study, we identified their vegetation as belonging to the *Ammophiletea* class and the *Koelerio glaucae-Corynephoretea canescentis* class, respectively. In the paper by Peyrat and Fichtner (2011), who surveyed dune vegetation of the southern Baltic coast, communities representing the latter class were generally more species-rich than those representing the former class. This is the opposite of our case. The probable reason for this discrepancy is that our communities are, in fact, late succession sub-stages of the above-mentioned classes. In other words, the yellow dune vegetation in this study should be classified as belonging to the *Elymo-Ammophiletum arenariae festucetosum* subassociation (Matuszkiewicz 2013), which is closely related to the grey dune vegetation. Similarly, the grey dune vegetation, being influenced by nearby Scots pine forest, is to some extent close to the species-poorer vegetation of brown dunes. The discrepancy may also result from the fact that, when calculating the total species richness of dune communities, Peyrat and Fichtner (2011) included vascular plants, bryophytes and lichens, while in our study, the latter two groups – known to be more abundant in grey dunes than in yellow dunes (Isermann 2008b; Peyrat and Fichtner 2011) – were not surveyed.

Invasion-induced changes in open dune vegetation and soil

The plots invaded by *R. rugosa* differed significantly from the non-invaded plots in terms of resident vegetation characteristics. The strong interaction effect, omnipresent in the results of the statistical analyses, indicates that the nature of these differences depended on the type of plant community or, more broadly, the stage of the dune succession. The impact of *R. rugosa* on the quantitative parameters of resident plant communities, i.e. the total cover and species richness, turned out to be fully in line with our expectations: it was negative, but only visible within the yellow dunes. A probable explanation for this phenomenon is that the species of the early succession stages adapt primarily to the challenges of the abiotic environment and not to intense interspecific competition for resources (Olff et al. 1993; Callaway and Walker 1997). As such, they stand no chance against invaders, such as *R. rugosa*, which are strong competitors and, at the same time, perform well in harsh environmental conditions (Bruun 2005). The same cannot be said for the species of the later stages of succession. They have a greater ability to compete, as evidenced by the very fact that they are able to replace existing species in the course of succession (note that quantitative parameters of the grey dune resident vegetation did not decline as a result of invasion; in the case of species richness, the tendency was even the opposite).

The compositional shift in the resident vegetation was another effect of the *R. rugosa* invasion. It was limited to the grey dune community and resulted from significant declines in the frequency and abundance of its two important components, *Corynephorus canescens* and *Ammophila arenaria* and a marked increase in the presence of *Festuca villosa*. The observed response of *Festuca villosa* to the invasion is quite an

intriguing phenomenon. This salt-tolerant grass is adapted to pioneer unstable sandy ground and is, therefore, abundant in yellow dune communities. However, being sensitive to competition, it disappears from these communities when invaded. Thickets of *R. rugosa* in the grey dune habitat theoretically offer even less favourable conditions for *Festuca villosa*, yet our field records show quite the opposite; *Festuca villosa* is a constant component of this community (cf. Suppl. material 1: table S1). This dual behaviour of *Festuca villosa* may be due to the presence of some ecotypic variation across populations of this plant in the study area – one ecotype typically occurs on the early succession dunes and the other in more closed habitats. This supposition is supported by the fact that *Festuca villosa* (synonym: = *Festuca rubra* ssp. *arenaria*) belongs to *Festuca rubra* agg., which is an ecologically diverse grassland-forest taxon, known for its innate adaptability to different environmental conditions (Rozema et al. 1978; Rhebergen and Nelissen 1985; Dąbrowska 2011). The consequence of this reasoning is that *R. rugosa* accelerates ecological succession in grey dunes by facilitating the entry of "forest" plants into the area of originally grassland vegetation. A slight increase in the presence of *Polypodium vulgare* (which is essentially a forest species) in the invaded grey dune communities is in line with the above conclusion.

R. rugosa, as a potential transformer species, may affect resident vegetation indirectly by changing habitat conditions. We expected the direction and magnitude of such changes to be similar for both types of dunes, with more pronounced community-level consequences in the case of yellow dunes (since plant communities of the early stages of succession function under a shortage and sometimes excess, of many resources, they may be more susceptible to fluctuations in their level than communities of milder environments). The obtained results did not confirm this hypothesis. Only two of the measured soil parameters (total N and phosphate contents) changed as expected both in the yellow and grey dunes (they increased under *R. rugosa*), without, however, causing a clear response of resident plants (for example, in form of the appearance of nutrient-demanding species). The remaining soil parameters either did not differ between the invasion and control plots or they differed only within the grey dune sites. Amongst the latter, noteworthy is the thickness of the organic layer, which doubled in the invasion plots. Varying wind exposure likely contributed to this pattern. Grey dunes are located further from the sea, in places sheltered on one side by other dunes and, on the other, by forest, which translates into less intensive blowing of the litter produced by *R. rugosa* and faster accumulation of organic matter in the soil. Perhaps this is part of the mechanism that facilitates brown dune species entry into the grey dune communities discussed above.

Open dune vegetation response to the variability of invader thickets

A meta-analysis of data from observational studies and experimental manipulations clearly showed that the competitive pressure of the invasive species is a function of its abundance (Sofaer et al. 2018; Bradley et al. 2019). Regardless of the shape of this function, it can be expected that the negative impact of invasion on resident communities

will initially increase, that is, as the invader spreads. However, after a longer period of time, this time-impact relationship may become weaker or even reversed, as demonstrated in the review by Strayer et al. (2006). The reasons for this phenomenon can be both external and internal. The former includes changes in the biological community and/or the abiotic environment, for example, the emergence and proliferation of pathogens or predators (Fan et al. 2016: Flory et al. 2018) and the latter changes in the invader, for example, its ageing (Staska et al. 2014). Both may reduce the density and vigour of the invader, thus facilitating the recovery of resident species.

R. rugosa is a perennial plant, so it is common to find individuals representing different ontogenetic stages within the area of invasion. At the latest stage, when the plant ages, dead shoots appear and, with them, gaps in the canopy. We expected that the resulting reduced competitive pressure would have a positive effect on the occurrence of resident plants. However, we did not observe any relationship between the variables reflecting the developmental stages of *R. rugosa* (in particular, the percentage of dead shoots) and the quantitative and qualitative parameters of the resident plant community. It is possible that the sampling criteria adopted in the study contributed to this result. The plots were established more or less in the middle of the *R. rugosa* patches, i.e. in their older parts, thus excluding the younger specimens, which dominated at the edges, i.e. at the front of the invasion. Consequently, the ontogenetic variation of *R. rugosa* could not be fully captured.

Interestingly, the total content of chlorophyll in *R. rugosa* leaves turned out to be an important explanatory factor for the resident plant community parameters. Regardless of the type of dune, its low values were usually accompanied by high cover and species richness of resident vegetation, as well as more abundant occurrence of species characteristics of dune grassland communities. The chlorophyll content is considered an indicator of a plant's nutrient supply or exposure to environmental stresses (Zhang et al. 2022). Therefore, the result suggests that there is a gradient of habitat severity within both the yellow and grey dune sites affecting *R rugosa*. Even if this gradient is not visible in the abundance of the invader, it is reflected in its condition (and, thus, in its competitive potential), which creates an opportunity for resident vegetation to recover.

Conclusions

R. rugosa readily invades both yellow and grey dune communities on the Baltic coast. Significantly, although these communities are closely related and often occur next to each other (they represent two adjacent stages of ecological succession), *R. rugosa* affects them in a radically different way. In yellow dune sites, it outcompetes most of the resident plant species, which is reflected in substantial declines in their total cover and richness. Given that there are virtually no changes in soil properties due to invasion, the underlying mechanism is likely to include only direct effects, such as space takeover and shading. In grey dune sites, *R. rugosa* causes a shift in the species composition,

without altering the quantitative parameters of the resident community. Contrary to the yellow dune situation, the presence of *R. rugosa* has a significant impact on the soil – the litter it produces can accumulate (because the grey dune habitat is less exposed to the wind), thus creating a solid organic layer. Shading (direct effect) and habitat transformation (indirect effect) appear to promote plants of brown dunes (for example, shade-tolerant ecotype of *Festuca villosa*, *Polygonum vulgare*), which means that the *R. rugosa* invasion of grey dunes accelerates their ecological succession. Finally, the effect of invasion depends not only on the type of habitat, but also on the invader itself. When the condition of the invader is degraded, for example, as a result of environmental stresses, there is an opportunity for resident plants to recover.

The above findings confirm the main hypothesis put forward in this paper, namely that the influence of *R. rugosa* on local vegetation and soil is modified by the ecological context. This context should be taken into account when, for example, invader removal measures are planned. It seems that such measures could bring better results in the yellow dunes. Firstly, because *R. rugosa* does not significantly change soil properties in this habitat. Secondly, mechanical disturbances to the soil profile that occur when removing invasive plants are probably of little importance in a situation where the soil surface is not stabilised anyway. In the case of grey dunes, in addition to removing the invader, it may also be necessary to remove excess organic matter. Moreover, measure-induced disturbances are likely to alter the nature of this habitat with unpredictable consequences for the ecosystem.

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Supplementary material 1

Detailed information and results of supplementary analyses on the structure of resident plant communities

Authors: Marcin W. Woch, Paweł Kapusta, Małgorzata Stanek, Katarzyna Możdżeń, Irena M. Grześ, Elżbieta Rożej-Pabijan, Anna M. Stefanowicz

Data type: occurrences, statistics

Explanation note: Venn diagram showing the number of species exclusive for and shared between four types of plots: yellow dune control and invasion plots and grey dune control and invasion plots. Table showing species shared between and exclusive for four types of plots – yellow dune control and invasion plots and grey dune control and invasion plots – with their frequency and average percentage coverage. Table showing resident plant species that contribute most to the dissimilarity between control and invasion plots according to the SIMPER analysis. Table showing resident plant functional traits for the control and invasion plots within the yellow and grey dune sites, and the effects of site type, plot type and their interaction on these properties, as shown by F-values derived from the LME analysis.

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RESEARCH ARTICLE

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Closely related invasive species may be controlled by the same demographic life stages

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Abstract

Invasive species that are closely related to each other may have similar population dynamics and, therefore, be controlled by targeting similar life stages. We studied two invasive knapweed species, spotted knapweed (*Centaurea stoebe* subsp. *micranthos*) and the hybrid meadow knapweed complex (*Centaurea* × *moncktonii*) in New York, USA, to determine their individual population growth rates (λ) across several sites over three years. Both knapweed species had growth rates that were greater than 1 (spotted knapweed λ ranged from 1.005–1.440; meadow knapweed λ ranged from 1.541–2.408), but there was high variability between years and sites. One study population of meadow knapweed was composed primarily of individuals of black knapweed ancestry (*C. nigra*), a species that, while introduced, is not invasive. For this population, the projected dynamics were stable (λ approximately 1). Elasticity analysis showed that the flowering-to-flowering stage contributed the most to population growth rate for six of seven sites and three additional transitions were also influential for four of seven sites of spotted and meadow knapweed: the seedling-to-vegetative stage, vegetative-to-flowering stage and flowering-to-seedling stage. We simulated how increasing vital rates would affect population growth and found that both spotted and meadow knapweed followed the same pattern. The vital rate of established seedlings maturing to flowering plants had the greatest effect on population growth, followed by the survival of new and established seedlings. In all cases, the responses were non-linear, with small initial changes having a large effect. Increases in the vital rates of later stages also tended to have a positive effect on growth rate, but the effects were more modest. Although the sensitivity analysis indicated that early vital rates had the largest effect on population growth, targeting these stages is not practical for management. Rather, reducing older life stage survival or delaying maturation of vegetative individuals would be more effective. The similarity between the population dynamics and how each life stage contributes to population growth provides support that protocols developed for one species should be effective for the other species with the caveat that any biological control agent should be directly tested on the target species before being utilised.

Keywords
Biocontrol agents, biological invasions, *Centaurea* × *moncktonii*, *Centaurea nigra*, *Centaurea stoebe* subsp. *micranthos*, elasticity analysis, knapweed, population demography, population growth rate

Introduction

Invasive species have been increasing around the world and some of the worst invasive plant species invade agricultural fields and abandoned pastures (Müller-Schärer et al. 2018). The proliferation of invasive plant species into agricultural fields and meadows has caused economic damage and may require active management to remove or prevent further spread. Much of the focus on determining what makes species invasive has been on species traits such as plant height, seed size and growth rates (Westoby 1998; Drenovsky et al. 2012). Traits alone are often not sufficient to predict which species will become invasive (Catford et al. 2019); rather, invasiveness can be better predicted by the maximum population growth rate and how growth rate is altered by environmental conditions (Palma et al. 2021). However, far fewer studies examine population growth rate compared to those that measure traits thought to predict invasiveness, in part because data collection for demography studies is often more time consuming (Palma et al. 2021). To document population dynamics in invasive plants requires conducting a census of life history stages over several seasons to estimate the transitions between stages (i.e. growth and survivorship), as well as detailed measures of stage-specific fecundity (Milbrath et al. 2018). Variation in climatic conditions yearly can have large effects on the demographic transitions (Elderd and Doak 2006). Furthermore, invasive populations can experience very different demographic transitions at a local scale depending upon environment or habitat conditions, the land-use history of the area and the stage in the introduction history of the species, making it necessary to sample at multiple locations over several years to obtain meaningful estimates (Jongejans et al. 2010).

Uncovering the population dynamics of invasive populations through demographic studies has the additional benefit of providing information for control strategies that target specific life stages that contribute most to the population growth rate (Hansen and Wilson 2006). Detailed demographic data are critical for determining the demographic transitions between stages that have the greatest impact on population size so that these stages can be the target of population control efforts (Parker 2000). Determining the growth stages that will limit the population growth rate is also critical for matching invasive populations with potential biological control agents (Milbrath et al. 2018).

Matrix population models can be paired with detailed demographic data to estimate a population's projected growth rate (Caswell 2001). Detailed demographic studies may not be possible for most species, so determining whether closely-related species are regulated by the same demographic stage is helpful for determining management and control of populations. However, species with shared evolutionary histories and similar traits may undergo different population dynamics (Ramula et al. 2008) and

the contribution of each life stage to the population growth rate may also differ (Jelbert et al. 2019). Elasticity analysis provides a mechanism whereby one can examine how changes in one life stage affect population growth, while holding the other stages constant (Caswell 2001). Such studies allow for management to target the life history stages that will achieve the greatest reduction in population growth (Benton and Grant 1999). While average population growth rates can be useful for comparing amongst similar species and sites, year to year variability may make it challenging to predict projected population growth rates and, hence, management strategies (Akin-Fajiye and Gurevitch 2020). Year to year variability can be difficult to quantify in short term studies, but by having multiple plots in different sites, one can tease apart the larger climatic signal from the intra-site variation (Elderd and Doak 2006).

Knapweeds are an important genus of plant invaders that are short-lived perennials that grow abundantly in meadows and agricultural fields and include several species introduced to North America that have been classified as invasive (Coombs et al. 2004; Milbrath and Biazzo 2020). Spotted knapweed [*Centaurea stoebe* subsp. *micranthos* (Gugler) Hayek], originally from southeast and central Europe, has been present in the USA for over 100 years and spread throughout the eastern USA along disturbed ruderal habitats and railroad corridors (Broennimann et al. 2014; Akin-Fajiye and Gurevitch 2018). Meadow knapweed (*Centaurea* × *moncktonii* C.E. Britt.) has been increasing in the northeastern US (Milbrath and Biazzo 2020). Meadow knapweed originated as a hybrid between two other knapweed species, brown knapweed (*Centaurea jacea* L.) and black knapweed (*Centaurea nigra* L.) (Roché and Roché 1991). While both the parental species are introduced and sometimes classified as invasive, the hybrid species, meadow knapweed is more invasive than either of the parental species (Roché and Roché 1991). In this paper, we investigate whether spotted knapweed and meadow knapweed, two species with similar life histories and invasive behaviour, have similar projected population growth rates and whether the same life stages contribute to their population growth. Specifically, we ask:

1) What is the projected population growth of spotted knapweed and meadow knapweed and how variable are the growth rates between species, years and sites?
2) Which vital rates contribute the most to the population growth rates of each species and are the same vital rates important across species, years and sites?

Methods

Study species characteristics and invasion history

We measured vital rates in multiple populations of both spotted knapweed and meadow knapweed in western and eastern New York, USA. Both spotted and meadow knapweed are herbaceous short-lived perennial species with similar life histories. Spotted

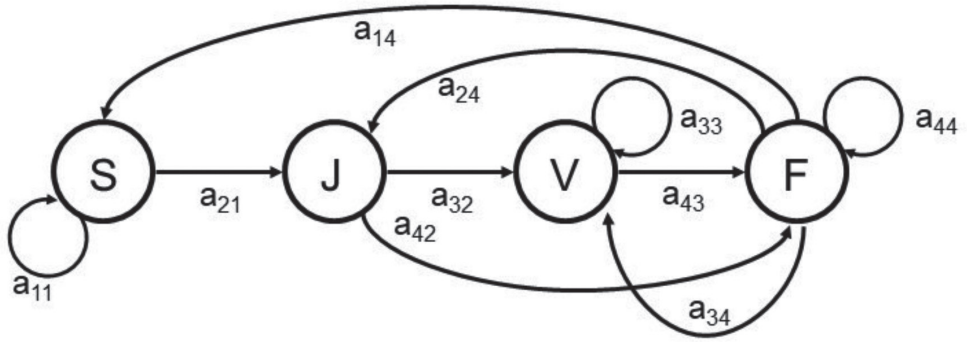
knapweed reproduces by seeds and plants typically flower in their second year; most flower every year thereafter (Emery and Gross 2005). Meadow knapweed reproduces by seeds and then the plants overwinter as a rosette of leaves; first flowering can take several years, but then plants usually flower annually thereafter (Roché and Roché 1991; Milbrath and Biazzo 2020).

Spotted knapweed is currently found in 46 U.S. States and six Canadian Provinces and was introduced from Europe in contaminated alfalfa (*Medicago sativa* L.) and soil ballast (Sheley et al. 1998). It has been a major rangeland and pasture weed in western North America and is an increasing problem in drier sites in eastern states including Arkansas, Michigan and New York (Winston et al. 2012; Carson and Landis 2014; Minteer et al. 2014; Akin-Fajiye and Gurevitch 2018). Meadow knapweed is found in 26 U.S. States and five Canadian Provinces (Keil and Ochsmann 2006; Poindexter et al. 2011; Winston et al. 2012) and is abundant in New York State and increasing in Vermont. Meadow knapweed is a fertile hybrid between black knapweed and brown knapweed (Hardy et al. 2000). Due to the hybrid nature of meadow knapweed and the variability in its traits, populations may consist of one, or both parental species plus the hybrid, creating a hybrid swarm (Lachmuth et al. 2019). Consequently, some putative meadow knapweed populations can contain substantial genetic contribution from one or both original parent species.

Data collection and analysis

We monitored three spotted knapweed populations in western New York, three putative meadow knapweed populations in west-central New York and one meadow knapweed population in eastern New York (Milbrath and Biazzo 2020; Suppl. material 1). The spotted knapweed populations grow on drier, well-drained soils, while the generally moist meadow knapweed sites were either abandoned pastures, grazed or still in active hay production (Milbrath and Biazzo 2020). The widely distributed fly *Urophora quadrifasciata* (Meigen) and the weevil *Larinus* sp. were present at all knapweed sites, which reduced the seeds present for both knapweed species to an unknown, but limited extent. Genetic identities of knapweed populations were verified through genomic analysis for meadow knapweed (Lachmuth et al. 2019), but not for spotted knapweed. After meadow knapweed populations were set up for monitoring, one of the sites (Jacobson) was identified as having predominantly genetic ancestry from black knapweed (*C. nigra*) (Lachmuth et al. 2019). Thus, our final population monitoring consisted of three spotted knapweed populations, three meadow knapweed populations and one putative predominantly black knapweed population (Suppl. material 1).

We designated four different life stages for monitoring and collection of demographic data: seeds in the soil seed bank (S), seedlings (J), vegetative plants (V) and flowering plants (F) (Fig. 1; Milbrath and Biazzo 2020). For purposes of the population modelling, 10 vital rates of germination, survival of life stages, transitions to other life stages and fecundity were primarily measured annually from August to the following August (usually before seed dispersal) for three transition years (2016–2019)



	S ($a_{.1}$)	J ($a_{.2}$)	V ($a_{.3}$)	F ($a_{.4}$)
S ($a_{1.}$)	$s_s(1-g)$	0	0	$fs_s(1-g)$
J ($a_{2.}$)	$s_s g s_{sj}$	0	0	fgs_{sj}
V ($a_{3.}$)	0	$s_{jv}(1-m_{jf})$	$s_v(1-m_{vf})$	$s_f r_{fv}$
F ($a_{4.}$)	0	$s_{jv} m_{jf}$	$s_v m_{vf}$	$s_f(1-r_{fv})$

Figure 1. Life-cycle diagram for the spotted and meadow knapweed populations and the associated population projection matrix. S = seeds in seed bank, J = seedlings, V = vegetative individuals, F = flowering plants. Arrows (a_{ij}) represent one-year transitions from August of year t to August of year t + 1 composed of vital rate combinations (shown in projection matrix; defined in Suppl. material 2). Matrix columns correspond with the stage ($a_{.j}$) from which individuals are transitioning at time t and rows indicate the stages ($a_{i.}$) to which individuals are transitioning at time t + 1.

(Fig. 1; Suppl. material 2). In total, ca. 42,000 seeds, 22,160 germinated seedlings and 7600 other life stages were marked and/or observed using a combination of germination trays and different-sized quadrats. Details of the population monitoring can be found in Milbrath and Biazzo (2020). We calculated vital rates for each developmental stage for each species and year at each site separately (Suppl. material 3).

Using the calculated vital rates, we derived transitions for all demographic stages by site for each year for each species (see transition matrix in Fig. 1). For example, flowering plants produced seeds (f), dispersed in late summer that, over the course of the next year, may remain dormant and enter the seed bank to increase the subpopulation of seeds, less any seed death (s_s) or reduction in seed numbers due to germination (1-g) that same year (transition a_{14}). A portion of other seeds may germinate to produce seedlings (g) although only a portion of those will survive to the following August (s_{sj}) to increase the subpopulation of seedlings (transition a_{24}). For each transition matrix, we projected the population growth rate (λ) at each site and year with starting values for each development stage at year 0 (2016). We also generated λ per site, based on the

average transitions of the three years of the study. For both sets of λ, we tested λ across all plots of each site for statistical differences from 1 (no change in population size) using Wilcoxon rank sum tests (α = 0.05).

We also performed an elasticity analysis (Caswell 2001). For the elasticity analysis, we tested the sensitivity of λ for each species to proportional changes in vital rates. We computed λ for each transition matrix derived from varying values of each vital rate while retaining the other values at their mean values across years and sites. We changed the vital rates by 1% for 100 steps, except for flowering where we changed the number of seeds by intervals of 1000 seeds (Fig. 4). To determine how the transitions between developmental stages influenced λ, we averaged the transition matrices for each site and species and year to derive the elasticities for the transitions between development stages.

All computations were performed in R, in particular using the packages dplyr (Wickham et al. 2018) and reshape2 (Wickham 2017) for data organisation, ggplot2 (Wickham 2009) for visualisations, vegan (Oksanen et al. 2019) for permutation-based similarity tests and popbio (Stubben and Milligan 2007) for demographic analyses.

Results

The average population growth rate (λ) was significantly greater than 1 for two of the seven sites, indicating that these sites had expanding populations (Fig. 2). For the three spotted knapweed sites (Black Pond, McEnteer, Wehle), the average λ ranged from 1.005–1.440, while for the three meadow knapweed sites (FLNF or Finger Lakes

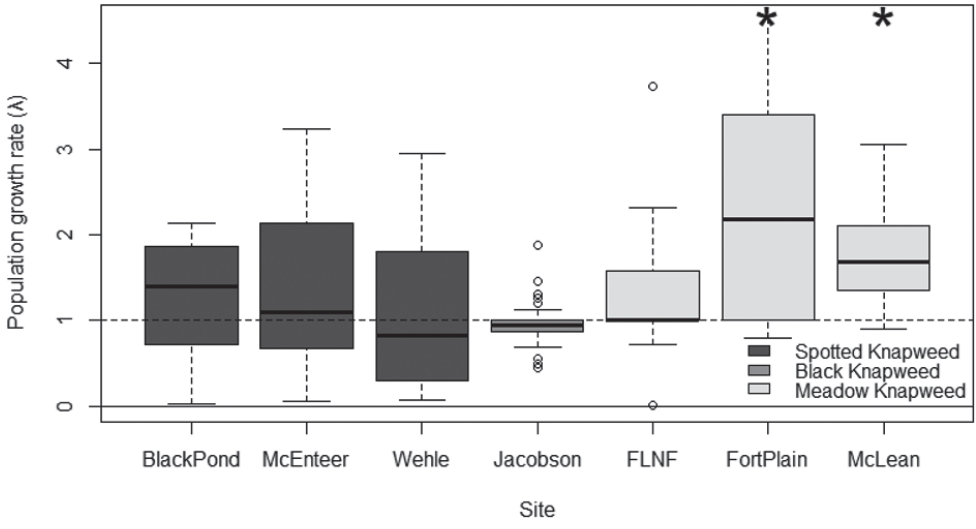


Figure 2. Population growth rate (λ) of knapweed species in each of the seven sites. Growth rates are based on the average transitions over three years. An asterisk shows population growth rates across all plots and years that were significantly greater than 1.0 ($P < 0.05$), with the dotted line indicating stable populations (λ = 1).

Table 1. λ and elasticities of λ for spotted knapweed, black knapweed and meadow knapweed, per location.

	Seed (S)	Seedling (J)	Vegetative (V)	Flower (F)	λ
Spotted Knapweed – BlackPond					
Seed	0.034	0	0	0	1.233
Seedling	0	0	0	**0.161**	
Vegetative	0	**0.161**	**0.173**	0.038	
Flower	0	0	**0.199**	**0.234**	
Spotted Knapweed – McEnteer					
Seed	0	0	0	0	1.440
Seedling	0	0	0	**0.220**	
Vegetative	0	**0.201**	0.102	0.087	
Flower	0	0.018	**0.288**	0.083	
Spotted Knapweed – Wehle					
Seed	0	0	0	0	1.005
Seedling	0	0	0	0.120	
Vegetative	0	0.099	0.014	0.007	
Flower	0	0.022	0.106	**0.632**	
Black Knapweed – Jacobson					
Seed	0	0	0	0	0.968
Seedling	0	0	0	0.048	
Vegetative	0	0.048	0.067	0.038	
Flower	0	0	0.086	**0.713**	
Meadow Knapweed – FLNF					
Seed	0	0	0	0	1.541
Seedling	0	0	0	0.079	
Vegetative	0	0.079	0.114	0.004	
Flower	0	0	0.082	**0.642**	
Meadow Knapweed – FortPlain					
Seed	0	0	0	0	2.408
Seedling	0	0	0	**0.203**	
Vegetative	0	**0.188**	0.030	0	
Flower	0	0.015	**0.189**	**0.375**	
Meadow Knapweed – McLean					
Seed	0	0	0	0.001	1.754
Seedling	0.001	0	0	**0.207**	
Vegetative	0	**0.208**	0.068	0.012	
Flower	0	0	**0.220**	**0.285**	

Bold numbers indicate highest elasticity values (> 0.15) for each population.

National Forest, Fort Plain, McLean), the average λ was higher, ranging from 1.541–2.408 (Table 1). In contrast, the Jacobson site with black knapweed genetic ancestry experienced a slight decrease in population growth, with the average λ being 0.968.

Average growth rates can obscure important variability that occurs between species at each site and year. For spotted knapweed, two sites (McEnteer and Wehle) showed similar patterns of population growth (Suppl. material 4, Fig. 3) characterised by λs below 1 in the first year, indicating declining populations. However, in year 2, the λs were significantly higher than 1 at both sites, indicating populations were growing. In Year 3, both sites had λs lower than 1 indicating populations were declining or stable; however, the results were not statistically different from 1, indicating uncertainty of the

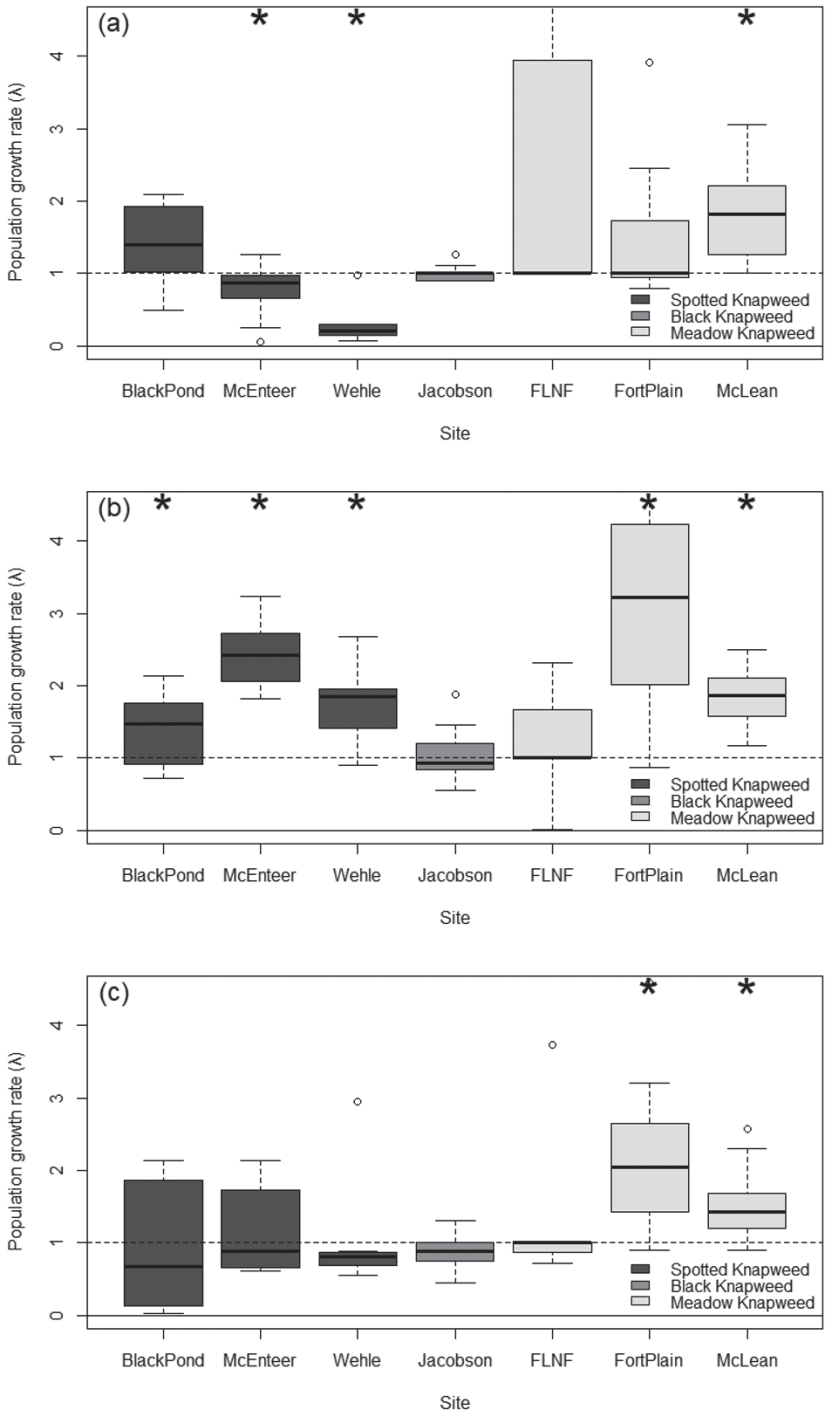


Figure 3. Population growth rate (λ) of knapweed species in each of the seven sites per year. Growth rates are shown for an August to August transition for **a** 2016–2017 **b** 2017–2018; and **c** 2018–2019. An asterisk shows population growth rates across all plots significantly greater than 1.0 ($P < 0.05$), with the dotted line indicating stable populations ($\lambda = 1$).

population decline (Fig. 3). At the third site (Black Pond), λs were greater than 1 for years 1 and 2, but less than 1 for year 3, although none was statistically different from 1 (Fig. 3). This lack of significance reflects the high variance in the plot-based estimates.

For meadow knapweed populations, the three sites were different from each other in their population dynamics. At the McLean site, λ was significantly greater than 1 for all three years of the study (Fig. 3). At Fort Plain in year 1, λ did not differ from 1, but it was significantly greater than 1 in the two subsequent years. In particular, the population grew markedly in year 2 with an average λ of 3.191, followed by another year of high growth rates (average λ = 2.202) (Suppl. material 4). At the FLNF site, λ was not different from 1 during any of the three years of the study (Fig. 3). However, in year 1 and year 2, there was high variability in the estimated λs. Population growth rates at the Jacobson site with black knapweed ancestry hovered near 1 each year with low variation in the estimated growth rates (Fig. 3).

Elasticity analyses allow for a determination of how life stage transitions contribute to the population growth rate. For six of seven sites, the flowering-to-flowering stage (a_{44}) had the largest elasticity, contributing 23 to 71% to the population growth rate (Fig. 1, Table 1). The single exception was the McEnteer site where the flowering-to-flowering transition had little effect on the population growth rate. For three of these sites (spotted knapweed-Wehle, meadow knapweed-FLNF, putative black knapweed-Jacobson), the flowering-to-flowering transition contributed 63% or more to λ, while other transitions contributed little (Table 1). For the remaining four sites of both spotted and meadow knapweed, three additional transitions had roughly similar and large contributions to the population growth rate: seedling-to-vegetative stage, a_{32}; vegetative-to-flowering stage, a_{43}; and flowering-to-seedling stage, a_{24} (Fig. 1, Table 1). These three transitions each contributed between 16 to 29% to population growth rate and involved seven of the ten vital rates measured (Fig. 1, Suppl. material 2). An additional transition, the vegetative-to-vegetative stage (a_{33}), was only important for the spotted knapweed site of Black Pond (Table 1).

We simulated how a change in each vital rate would affect population growth (Fig. 4). Not surprisingly, one vital rate (reversion from the flowering stage to a vegetative stage, r_{fv}) showed a decline in λ with an increased proportion of reversion. The remaining vital rates showed a slight to substantial increase in λ with an increase in the vital rate (Fig. 4). Spotted knapweed and meadow knapweed showed similar patterns (curves) for most vital rates, except for new seedling survival (s_{sj}) and fecundity (f). In the latter two cases, spotted knapweed may be more sensitive to changes in seedling survival and fecundity than meadow knapweed as indicated by steeper and diverging curves (Fig. 4). The vital rate that had the largest effect on λ was the seedling-to-flowering maturation rate (m_{jf}), whereas three vital rates had relatively little effect on λ for spotted and meadow knapweed: seed survival (s_s), survival of flowering plants (s_f) and the flowering-to-vegetative stage transition (r_{fv}) (Fig. 4). The remaining six vital rates appeared to have similar effects on λ. Notably, the putative black knapweed population responded differently from the two other knapweed species (Fig. 4). For this population, a decrease in the survival of flowering plants and an increase in the flowering-to-vegetative stage may reduce λ below 1 (Fig. 4).

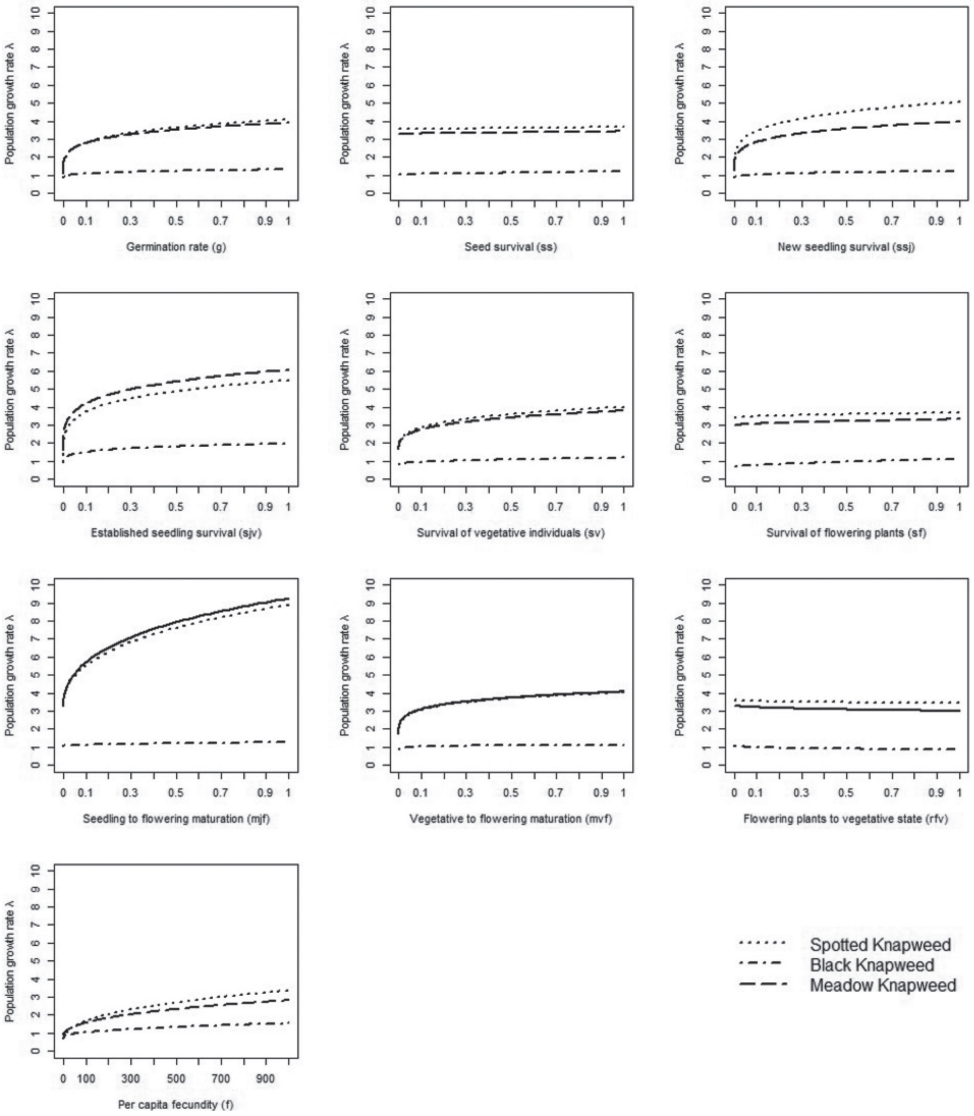


Figure 4. Sensitivity of each knapweed species' population growth rate to changes in specific vital rates.

Discussion

The increasing prevalence of spotted knapweed and meadow knapweed in the eastern USA makes understanding the population dynamics and the demographic transitions that contribute to their expansive growth rate important. Both spotted and meadow knapweed had average population growth rates (λ) greater than 1, indicating most of their populations are increasing in the Northeastern US and are capable of further spread and invasion. Although no similar studies on meadow knapweed exist, there are several studies that have projected the population growth of spotted knapweed (Emery

and Gross 2005; Maines et al 2013; Akin-Fajiye and Gurevitch 2020). In Michigan, Emery and Gross (2005) determined the average population growth was greater than 1 (1.17), but found variation amongst years with one year having a growth rate less than 1. Akin-Fajiye and Gurevitch (2020) determined population growth rates from experimental populations of spotted knapweed on Long Island, New York. In their study, the projected population growth of spotted knapweed ranged from 2.14 under high density and undisturbed conditions to 3.64 under low density conditions with disturbance. In our spotted knapweed populations, population density was high (Milbrath and Biazzo 2020) and, thus, the lower growth rates are consistent with Akin-Fajiye and Gurevitch (2020). Maines et al. (2013) projected population growth for spotted knapweed around Boulder, Colorado and found rates ranging from 1.3 to 1.7, with higher growth rates associated with higher precipitation.

As found in other studies (Emery and Gross 2005; Akin-Fajiye and Gurevitch 2020), we found large variation in λ amongst years. For spotted knapweed populations, two of the three populations appeared to alternate between low and high growth rates, a pattern consistent with a strong density-dependent response. This is consistent with the density-dependent response reported by Emery and Gross (2005) where lower growth rates were found with higher population densities. While density-dependence may play a role in the long-term dynamics of spotted knapweed, the short timeframe of our study makes it impossible to conclude that the populations were cycling between low and high densities. Moreover, environmental stochasticity and demographic stochasticity could also result in variable projected growth rates. For example, during our study, we observed a severe drought in 2016 that reduced spotted knapweed survival at the Wehle site. Similarly, a large population of *Larinus* weevils at the McEnteer site in 2018 was correlated with reduced seed production and subsequent low seedling recruitment (Milbrath and Biazzo 2020). However, the degree to which these and other unidentified factors are influencing specific populations is unclear.

For meadow knapweed, population growth rates were overall higher than for spotted knapweed (excluding the population composed primarily of black knapweed individuals). Both meadow knapweed and spotted knapweed populations showed overall high within-population variation in growth rates. Previous studies of the same meadow knapweed populations have shown high phenotypic variation in capitula traits (Lachmuth et al. 2019) and morphological traits (Molofsky, unpublished data). Genetic studies on the same population found that these meadow knapweed populations are advanced generation (beyond F2) recombinant hybrids and, thus, would be expected to express high levels of individual phenotypic variation (Lachmuth et al. 2019). The high phenotypic variance in hybrid meadow knapweed seems to result in a similarly high variance in vital rate parameters and projected population growth. Our previous genetic work also determined that one of our sites (Jacobson) had a high proportion of individuals with black knapweed genetic ancestry (Lachmuth et al. 2019). This site was projected to have an average population growth that is less than 1 with low variance across each site and across all years. This might suggest that populations composed of individuals with high amounts of black knapweed genetic ancestry may be declining; yet with just a single site with this ancestry background, this hypothesis must await

further investigation. Herbivory by slugs and snails was also high at this site (as well as other meadow knapweed sites); thus, subsequent mortality of earlier life stages undoubtedly influenced population growth, although to what degree is unknown (Milbrath and Biazzo 2020).

Projecting long-term invasive spread from short term studies should be done cautiously. This is especially true in the context of biological invasions, which are characterised by non-equilibrium population dynamics that often reflect the invasion history and the length of time since the species has colonised a given location (Ramula et al. 2008). A recently colonised site experiencing low density dependence may initially experience extremely high growth rates that may eventually become stabilised as the population ages and density dependence becomes more important (Ramula et al. 2008). In our study, knapweed populations had been present for several years prior to the study (Milbrath and Biazzo 2020). Thus, we expect the population dynamics estimated for these sites should reflect established populations. In fact, the relatively high density of plants within our sites indicates that some populations may have been limited by density (Milbrath and Biazzo 2020). While some populations showed average population growth rates greater than 1, there was also high spatial and temporal variability and, at any site and year, the growth rate could be stable or declining (Fig. 3). Moreover, it is not clear how much additional spread into adjacent sites is occurring. In knapweed species, the seeds are present in capitula, which drop to the ground. Thus, the seeds should be contained locally, with occasional longer distance seed dispersal through animal and human movement. This indicates that the risk of current populations spreading to new sites would be limited without large disturbance and human movement of seeds. However, the spread of meadow knapweed can come not only through spread of seeds to new sites, but also through the spread of their genes through pollination of either black knapweed or brown knapweed or through backcrossing with a hybrid meadow knapweed (Lachmuth et al. 2019). In the northeastern US where meadow knapweed has increased recently, its increase may be due to both hybridisation creating more meadow knapweed populations and their higher invasiveness compared to the two parental species.

The elasticity analysis allows us to predict how changes in individual life stage transitions will impact population growth. In the northeastern US, the most influential transition was the proportion of flowering plants that survived to flower in subsequent years. Thus, reducing population growth rates below 1 may be achieved by increasing mortality of adult plants. Management of other stages contributing to other influential transitions may also be productive in reducing population growth, i.e. the production of seed and subsequent recruitment of new seedlings into the population (via the flowering-to-seedling transition), the survival of established seedlings to the vegetative juvenile stage in their second year of growth (seedling-to-vegetative transition) and vegetative individuals flowering the following year (vegetative-to-flowering stage). The vegetative-to-flowering transition involved both the maturation of vegetative juvenile plants that had never previously flowered, as well as previously flowered individuals that became non-flowering for a year (Milbrath and Biazzo 2020). It was not always possible to distinguish these two sources, so they were combined in our analyses. These

results are comparable to Emery and Gross (2005) and Akin-Fajiye and Gurevitch (2020) for spotted knapweed although they used a five- and three-stage model, respectively, instead of the four-stage model that we used. We separated seedlings from vegetative individuals (unlike Akin-Fajiye and Gurevitch (2020)) because we could accurately track seedling fates, but we only included one flowering adult stage (as opposed to Emery and Gross (2005)) because the variation in stem number amongst sites and species in our study did not clearly indicate additional logical subdivisions to use. In another study of spotted knapweed carried out in Montana, Jacobs and Sheley (1998), using sensitivity analysis with a different demographic modelling approach, also identified survival of juvenile and adult plants, a juvenile-to-adult transition and seed production as important. Thus, vulnerable transitions seem to be consistent across spotted knapweed populations in different regions of the USA.

Reducing the rate of the four transitions, alone or in combination, should reduce population growth of northeastern populations of spotted and meadow knapweed by targeting the specific vital rates integral to these transitions (Fig. 1, Suppl. material 1). In their meta-analysis of invasive plants, Ramula et al. (2008) suggested that reductions in growth, fecundity or combinations of reductions including survival should result in declining populations in short-lived species like knapweeds. Based on our results, this could involve increasing mortality of flowering individuals (flowering-to-flowering transition), reducing fecundity directly and/or increasing mortality of newly-germinated seedlings (flowering-to-seedling transition), reducing the survival of established seedlings (seedling-to-vegetative transition) or reducing the survival of vegetative individuals and/or delaying or preventing the flowering of vegetative plants (vegetative-to-flowering stage). While the established seedling-to-flowering maturation rate had a large effect on λ (Fig. 4), it was rare or did not occur amongst our sampled populations (0–5% rate, Milbrath and Biazzo (2020)) and, thus, would not be a realistic focus for control. Although not having a large effect as the previously discussed vital rates, the transition from flowering individuals back to the vegetative state was associated with lowering population growth rates (Fig. 4). Across years and sites, 19–40% of flowering spotted knapweed plants naturally reverted to a vegetative state, whereas 2–10% of meadow knapweed plants did (Milbrath and Biazzo 2020). Thus, mowing or haying fields that contain spotted and meadow knapweed may slow their spread.

The population growth trajectory as a function of changes in one life history transition while holding the rest of the life stage transitions constant allows us to hypothesise how environmental or genetic changes may result in altered population growth. For control purposes, it provides information about how altering a key life stage should affect a population's projected growth rate. In our study, we were also interested in understanding whether closely-related species with similar life histories would follow similar patterns. For all species, increases in fecundity had a large effect on population growth rates and the increase was similar across species, although spotted knapweed may be more sensitive to changes in fecundity. Reducing fecundity through targeted removal of seed heads and seeds, either through mechanical measures or biological control agents, may help reduce population growth rates and spread, but not necessarily cause population declines amongst the knapweed species. Biocontrol by seed head-infesting

insects alone, such as tephritid flies (*Urophora* spp.) and weevils (*Larinus* spp.), had not reduced densities of western USA populations of spotted knapweed despite seed reductions greater than 90% (Story et al. 2008). In our study, the natural occurrence of both the fly *U. quadrifasciata* and *Larinus* weevils may have suppressed the populations to an unknown degree, underestimating the projected increase for both species in the Northeastern US. However, it is unclear what impact these biocontrol agents will ultimately have on knapweed populations in the Northeast; data on insect densities and their per capita impact are currently lacking for this region of the country. This is particularly true for meadow knapweed that has larger flower heads and produces twice as much seed per head as spotted knapweed (Woods et al. 2008; Milbrath and Biazzo 2020). Myers and Risley (2000), using a simulation approach for the related diffuse knapweed (*Centaurea diffusa* Lamarck), predicted that a biocontrol agent must be able to kill later life stages of knapweed plants due to population-level compensation to reduced fecundity and/or seedling recruitment (early seedling survival). Other agents that fulfil this criterion are credited with declining spotted knapweed populations in Montana, specifically the root-boring weevil *Cyphocleonus achates* Fahraeus (Story et al. 2006). Similarly, for Colorado populations of spotted knapweed, the projected population growth rate from a combination of seed-head and root-boring insects was approximately 1 (Maines et al. 2013). Thus, reducing seed output and young seedling survival alone is not considered a viable approach to managing knapweed populations.

For spotted knapweed and meadow knapweed, the transition from established seedlings to mature flowering plants had the greatest effect on population growth, followed by the survival of new and established seedlings. In all cases, the responses were non-linear, with small initial changes having a large effect. Increases in the vital rates of later stages also tended to have a positive effect on growth rate, but the effects were more modest. Although the sensitivity analysis indicated that early vital rates had the largest effect on population growth, targeting these stages is not practical for management. Reducing older seedling, vegetative juvenile and adult survival or delaying maturation of vegetative individuals could limit population growth. Removing plants by mechanical means, such as pulling or through applications of herbicides, can aid spotted knapweed suppression and restoration efforts (MacDonald et al. 2019), but can be inefficient, time consuming and expensive. An alternative approach may be to identify a biocontrol agent that interferes at these stages. For example, the reported success of *Larinus minutus* Gyllenhal in reducing populations of diffuse knapweed in western North America (Winston et al. 2012) is apparently due to adult weevil feeding damage in addition to the expected impact from larval destruction of seeds. High densities of adult weevils can kill juvenile and adult plants through their feeding on rosette leaves and bolting stems (Myers et al. 2009). Such effects from *Larinus* adults have not been reported for spotted knapweed, but they do highlight the importance of post-seedling mortality that can be caused by agents such as *C. achates* (Story et al. 2006). Therefore, successful agents of western populations of spotted knapweed should be considered for use in the Northeast. However, it is unclear whether insect-induced plant mortality is effective as experimental assessments have usually involved the presence of multiple agents attacking different parts of the plant (Story et al. 2006; Maines et al 2013).

Further, Maines et al. (2013) suggested that other measures in addition to biocontrol would be needed to achieve a negative population growth rate of spotted knapweed in some locations, for example, a combination of appropriately-timed mechanical or chemical removal of plants that do not interfere with existing biocontrol agents. Given the similarity in the dynamics between northeastern populations of spotted knapweed and meadow knapweed, a similar approach could be applied to meadow knapweed. Although a few biocontrol agents released for spotted and diffuse knapweed control have naturally infested meadow knapweed in the Northwestern and Northeastern US (Coombs et al. 2004; Woods et al. 2008), new biocontrol agents may be needed as current successful agents may not survive or develop in the moister habitats containing meadow knapweed, they may not easily transfer or may have a smaller impact. In contrast, for the site with black knapweed ancestry, altering vital rates had minimal effect on overall population growth rates with the one exception being the survival of flowering plants. The low number of single origin populations found for both black knapweed and brown knapweed in the Northeast suggests extinction by hybridisation for these two parental species. Thus, management efforts and biocontrol strategies should be focused exclusively on the spotted and meadow knapweed populations.

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Supplementary material 1

Knapweed locations in New York State. FLNF = Finger Lakes National Forest

Authors: Jane Molofsky, Dominik Thom, Stephen R. Keller, Lindsey R. Milbrath

Data type: Location of study sites (table)

Explanation note: Information on the location of each study site.

Link: https://doi.org/10.3897/neobiota.82.95127.suppl1

Supplementary material 2

Lower-level vital rates for the knapweed matrix population model

Authors: Jane Molofsky, Dominik Thom, Stephen R. Keller, Lindsey R. Milbrath

Data type: table

Explanation note: Lower level vital rates used in the matrix population models.

Link: https://doi.org/10.3897/neobiota.82.95127.suppl2

Supplementary material 3

Vital rates for each knapweed species by each site and by each year

Authors: Jane Molofsky, Dominik Thom, Stephen R. Keller, Lindsey R. Milbrath

Data type: table

Explanation note: Vital rates for spotted knapweed, black knapweed and meadow knapweed per site and year of measurement.

Link: https://doi.org/10.3897/neobiota.82.95127.suppl3

Supplementary material 4

Elasticities by species by plot by site by year

Authors: Jane Molofsky, Dominik Thom, Stephen R. Keller, Lindsey R. Milbrath

Data type: table

Explanation note: λ and elasticities of λ for spotted knapweed, black knapweed and meadow knapweed per site and year of measurement.

Link: https://doi.org/10.3897/neobiota.82.95127.suppl4