

# Prioritisation of quarantine pest list for the Caribbean using a multi-criteria decision approach

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## Abstract

Quarantine plant pests are socially, economically and environmentally important due to their impact on food security, human health, global trade and crop production costs. The increase in global trade and tourism, frequent occurrence of natural disasters and climate changes have exacerbated the rate of entry, establishment and spread of plant pests regionally and globally. It has, therefore, become exigent to develop a list of pests of quarantine importance at the regional and national levels to prioritise and allocate the limited available resources to manage the associated risks. In the present study, the Technical Committee on the Formulation and Prioritisation of a Regional Priority Pest List for the Caribbean, in collaboration with the National Plant Protection Organisation of the Caribbean countries and the United States Department of Agriculture - Animal and Plant Health Inspection Service (USDA-APHIS), developed and prioritised a quarantine pest list using a multi-criteria decision-making approach. The technical

committee successfully evolved the process in 2014 and 2018 and developed a list of the top 10 pests of quarantine importance for the Caribbean Region, employing the Delphi Technique (DT) and Analytical Hierarchy Process (AHP) through the assignment of criteria that are relevant to the region. The Mediterranean fruit fly (*Ceratitis capitata*), frosty pod rot (*Moniliophthora roveri*) and the tomato leaf miner (*Tuta absoluta*), listed as top quarantine pest threats, were subsequently detected in the region. This exercise guided the authorities in advance to allocate resources and to develop response plans including capacity building for surveillance and detection of priority pests. This has demonstrated the significance and appropriateness of the multi-criteria decision approach to determine priority pest lists and prepare the region for development of better management practices.

### **Keywords**

Analytical Hierarchy Process, Delphi Technique, invasiveness, quarantine pest, economic loss

## **Introduction**

The Caribbean Region, characterised by tropical and sub-tropical agriculture, is well known for its diversity. Each country is unique in culture and food habits. Due to its rich diversity, the region has been listed in the world's 36 biodiversity hotspots with more than 1,500 unique plant species that are not present elsewhere in the world (Mittermeier et al. 2004, 2011). The region produces the popularly-known and sought-after fine flavoured cocoa, some of the hottest peppers in the world with more than one million Scoville Heat Units, high pungency ginger, richly flavoured coffee, Spice Islands 'nutmeg', as well as the premium quality starch from the St. Vincent arrowroot. A range of cereals (rice, corn), vegetables (tomato, hot pepper, cucurbits, cabbage, lettuce, legumes), roots and tubers (cassava, sweet potato, yam, taro), sugar-cane, coconut, spices, coffee, citrus, pineapple, plantain, banana, breadfruit and papaya are also produced for local consumption and export.

According to the Food and Agriculture Organisation of the United Nations (FAO), plants provide over 80% of the food consumed by humans and serve as the primary source of nutrition for livestock. It estimates that 40% of global crop production is lost to pests every year. Annually, plant diseases and invasive insects cost the global economy approximately US\$220 billion and US\$70 billion, respectively (IPPC Secretariat 2021; Ristaino et al. 2021). To date, over 10,000 fungal species associated with plants have been discovered and it is reported that fungal infections cause more harm than the diseases caused by other pathogenic micro-organisms (Hussain and Usman 2019; Nazarov et al. 2020). A viral infection can lead to 98% crop loss in tropical and sub-tropical regions (Czosnek and Laterrot 1997). Phytoplasma infections can significantly decrease both crop yield and quality. Crop losses to an extent of 40%, 60%, 93%, 30–80% and 100% were reported in eggplant, tomato, pepper, potato and cucumber, respectively specific to Phytoplasma diseases (Kumari et al. 2019). Similarly, plant-parasitic nematodes were reported to cause 12.3% crop losses with an estimated value of US\$173 billion per year (Kumar et al. 2020).

Many pests of quarantine importance were also reported in the Caribbean (CABI 2012). Lethal yellowing in coconut was first observed in the Caribbean in the late 1800s and continued to be a serious problem in the Caribbean and Central America

(Johnson 1912; Plavsic-Banjac et al. 1972; CARDI 2013). It was proposed that import of cattle fodder from India to the Caribbean would have carried the vector for Phytoplasma disease affecting palms (Ogle and Harries 2005; Gurr et al. 2016). Similarly, the Fusarium wilt fungus (*Fusarium oxysporum* f.sp. *cubense* race1) that devastated the Gros Michel Bananas variety in the region, might have been introduced into the Caribbean with the Silk banana variety that came from south India and, from there, spread to Central and South America (Blomme et al. 2013). The spread of the invasive hibiscus mealybug (*Maconellicoccus hirsutus*) and the red palm mite (*Raoiella indica*) had a serious impact on Caribbean agriculture. Between 1995 and 1998, an estimated total of US\$18.3 million was spent on the control of hibiscus mealybug (Edwards 1999). Although total economic losses due to the pink hibiscus mealy bug have not been computed, the cost in Grenada (1995–1998) included annual losses of an estimated US\$4.6 million. Amongst these was the cost of \$1.1 million for the control of the mealybug and the loss of 38 hectares of blue mahoe (*Talipariti elatum*). The cost in St. Kitts and Nevis, including the employment of management practices, was estimated to be US\$0.3 million. The potential loss to agriculture and forestry in Trinidad and Tobago was estimated to be US\$125 million. The total reported loss to the Caribbean was approximately US\$138 million – excluding control costs and loss of exports. It was estimated that the potential annual loss to the United States of America if the pink hibiscus mealy bug were established there, would have been US\$750 million (Moffitt 1999). The introduction of the Giant African Snail (*Achatina fulica*) from East Africa has been a menace in the Caribbean islands of Antigua, Barbados, Dominica, Saint Lucia and Trinidad (Pollard et al. 2008). Additionally, the Mango Seed Weevil (*Sternochetus mangiferae*) and Black Sigatoka Leaf Spot (*Mycosphaerella fijiensis*) are a few more examples of economically significant pests introduced into the Caribbean (Meissner et al. 2009).

Plant pests have also been a major contributing factor to the declining productivity of key plantation crops that contributed significantly to agricultural gross domestic products earning of foreign exchange and employment generation. This in turn contributed to significant decline in these major plantation crops. Some examples of these are Witches broom in Cocoa; Citrus Tristeza Virus and Huanglongbing (HLB) in citrus production; Lethal Yellowing and Red Ring in coconuts and Black Sigatoka in bananas. The increase in agricultural trade due to a huge reliance on food imports (valued at US\$5 billion), the high dependency of Small Island Developing States (SIDS) on tourism for their livelihood, the frequent occurrence of natural disasters in the region and the greater vulnerability of SIDS to climate change have intensified the chances of entry, establishment and spread of invasive pests in the region. The Caribbean Region has experienced serious economic, social and environmental challenges due to the intrusion of invasive pests (Pollard et al. 2008). When invasive alien species (IAS) are introduced into the novel habitat with enhanced survivability, they can cause widespread harm to both native and cultivated plant populations. The losses from damage and costs associated with management of established IAS could exceed the cost of measures to prevent introductions from occurring. In this regard, many National Plant Protection Organisations (NPPOs) around the world use a proactive approach through the implementation of trade restrictions as a strategy to

minimise the probability of introduction of IAS. NPPOs may also use various strategies to stay informed about pest species that may threaten their respective jurisdictions. In this context, it is important to prioritise the list of pests of quarantine importance and to design strategies for preventing the entry of exotic pests into the country. The strategies include monitoring, assessing and developing capacities to identify and diagnose at all levels, developing an early warning system and risk mitigation measures and developing a national pre-border, border and post-border response plan with continuous intelligence. Furthermore, the prioritisation process guides the national and regional authorities to prioritise and allocate resources towards the implementation of appropriate quarantine and phytosanitary measures (MacLeod and Lloyd 2020).

At the same time, inconsistencies in the randomised prioritisation process may negatively impact sound judgement leading to the oversight of the differences in potential outcomes and the high-risk factors. There is, therefore, a strong need for a standard, precise and rigid valuation process that minimises biases and accommodates multiple factors when prioritising regional pests. In this context, Delphi Techniques and the Analytical Hierarchy Process (AHP) were explored in the present study.

The Delphi method, named after the ancient Greek oracle, was developed by the research organisation RAND in the 1950s to apply research and development-based decisions to predict military actions. Of late, this technique has been popularly used in economics to gain consensus amongst anonymous experts by sharing a specific research question (de Villiers et al. 2005). This technique has been demonstrated to accomplish a convergence of opinion on a specific real-world issue. This has been successfully applied in the field of medicine, social policy, tourism and sustainability where specific information is limited and contrary. However, it is less commonly applied in ecology and conservation despite its suitability in dealing with biodiversity management issues that are equally complex and involve multiple stakeholders and trade-offs (Mukherjee et al. 2015). It has the advantage of developing a full range of alternatives, exploring or exposing underlying assumptions, as well as correlating judgements on a topic spanning a wide range of disciplines. The Delphi Technique was predicated on the rationale that “two heads are better than one, or n heads were better than one”. Common surveys often try to identify “what is important”, whereas the Delphi Technique attempts to address “what is priority” (Hsu and Sandford 2007).

Similarly, the AHP has been demonstrated as a successful tool that diligently handles the multi-criteria decision-making process. Due to increasing complex nature of multi-criteria decision-making approaches, especially in the fields of agriculture and biodiversity, the process of decision-making has become more critical and challenged nowadays (Kumar and Pant 2023). This technique has allowed the selection of the best alternatives that depends on multiple criteria and sub-criteria and potentially reduces the complications by making several comparisons amongst the elements of the hierarchy. It captures both subjectivity and objectivity of associated problems and provides an ongoing measure of the consistency of the decisions obtained. Based on analysing the merits and applications of these methods in the multi-criteria decision-making approach, the current study employed Delphi Technique and AHP to prioritise the list of pests of quarantine importance for the Caribbean.

## Materials and methods

### Technical committee on the formulation and prioritisation of a regional priority pest list

The technical committee of the Caribbean region was constituted in 2011. The committee was comprised of the regional subject matter specialists viz. an entomologist, fungal pathologist, virologist, bacteriologist, malacologists, nematologist, weed scientist and an agricultural economist. The committee employed the Delphi Technique and the Analytical Hierarchy Process to formulate and prioritise the regional pest list of quarantine importance in 2014 and 2018. Prior to the committee meetings, a series of virtual meetings and email transactions were held to share the quarantine list of national importance from the National Plant Protection Organisation of the Caribbean countries. These lists were consolidated for consideration and analysis by the committee.

### Delphi Technique in prioritisation of regional pest list

The Delphi Technique is used to estimate the likelihood and outcome of future events, based on expert opinion. It places a premium on “Expert Opinion” and uses qualitative information provided by reputable professionals working in a particular subject-matter area.

In this study, the National Technical Authorities were trained in the development of national pest lists, based on the traditional guidelines and International Standards for Phytosanitary Measures (ISPMs). The quarantine pest list of the Caribbean countries (Antigua and Barbuda, Dominica, Dominican Republic, Grenada, Jamaica, Guyana, Guadeloupe and Martinique, Trinidad and Tobago, Saint Lucia and Saint Kitts and Nevis) were reviewed firstly in the process of prioritising a regional pest list by the Regional Technical Committee. The following resources were consulted by the experts in shortlisting the regional pests.

1. The Centre for Agriculture and Bioscience International (CABI) Invasive Alien Species Compendium;
2. Caribbean Pathway Analysis (Meissner et al. 2009);
3. The CARICOM’s List of 19 Commodities of Importance;
4. The Agriculture Policy Programme, in which CARICOM identified three (3) commodities that include cassava, condiments, spices and herbs.

Each expert selected their top 10 insect pests, weeds, molluscs, fungi, bacteria, nematodes and viral pathogens, based on the following criteria:

- Invasiveness;
- Potential Spread Entry/ Exit Pathway;
- Impact on social systems;
- Economic domestic impact;

- Economic Trade Impact;
- Economic and Environmental goods Impact;
- Environmental impact;
- Feasibility of Management.

The details of factors considered for each criterion is given in Table 1. After the consultation process facilitated by the technical committee coordinator, the subject matter specialists presented the pest lists. Based on the opinion of the specialists, the top priority pest list was compiled.

## Analytical Hierarchy Process in prioritisation of regional pest list

To rank the pests of importance identified from the Delphi Technique (DT), the Analytical Hierarchy Process (AHP) was used in the current study. AHP is a multi-criteria

**Table 1.** Pest Prioritisation Criteria used by experts in the Delphi Technique.

Criteria	Factors
1. Invasiveness (establishment)	<ul style="list-style-type: none"> <li>• Pest has demonstrated invasive capability in new distribution outside of its natural range</li> <li>• Pest is distributed in climates similar to that throughout</li> <li>• Hosts of the pest are available and prevalent in the region</li> <li>• Reproductive potential is high (no. progeny/female; no. generations/year; asexual capability)</li> <li>• Dispersal capabilities</li> <li>• How invasive the pest (Ability to establish and thrive)</li> </ul>
2. Potential Spread Entry/ Exit Pathway	<ul style="list-style-type: none"> <li>• Pest is highly mobile (capable of flight; carried easily by wind, other organisms or moving water)</li> <li>• Natural barriers in country absent or not likely to prevent natural spread of the pest</li> <li>• Pest travels with commodities that are moved commonly by man</li> <li>• Documentation and factors to consider:               <ul style="list-style-type: none"> <li>○ high interception numbers</li> <li>○ hitchhikes on non-hosts</li> <li>○ frequently imported commodities are hosts</li> <li>○ hosts imported for planting</li> <li>○ Is smuggling likely?</li> <li>○ What have neighbouring countries recorded for these items?</li> </ul> </li> </ul>
<b>3. Impact on social systems</b>	<ul style="list-style-type: none"> <li>• Food security</li> <li>• Loss of employment</li> <li>• Human health</li> <li>• Livestock and pet health</li> <li>• Amenities</li> <li>• Heritage values</li> </ul>
4. Economic / domestic impact	<ul style="list-style-type: none"> <li>• Production cost, domestic market share</li> <li>• GDP considerations</li> <li>• Crop Loss / loss of primary production</li> <li>• Farmers cost of controlling or managing pest</li> </ul>
5. Economic / Trade Impact	<ul style="list-style-type: none"> <li>• Foreign trade / exports of goods</li> </ul>
6. Economic / Environmental goods Impact	<ul style="list-style-type: none"> <li>• Impact on tourism products – export of services and aesthetic value</li> <li>• Reduction in or limitation to indigenous species (flora and fauna)</li> <li>• Negative ecosystem changes</li> </ul>
7. Feasibility of Management	<ul style="list-style-type: none"> <li>• Public costs including surveillance, detection and control</li> </ul>

decision-making method that was developed and extensively studied by Thomas L. Saaty in the 1970s. It is grounded in mathematics and human psychology and has specialised application in group decision-making where a diversity of skills, knowledge and experiences are of particular value. The subject matter specialists used the recommendations from the DT to rank pests using the AHP. The AHP model, viewed as better suited to the development of the Caribbean pest list, was tailored by Seepersad and Ram (2011) and used in the current study. The AHP model is given in Suppl. material 1.

The process of ranking the plant pest list comprised of the stepwise processes: (i) identifying the criteria that were relevant to the region, based on the social (food security, human health), environmental (crop health, aesthetic value and ecosystem health) and economic importance (production costs, foreign trade and export earnings, public costs), (ii) developing a scale of importance for pairwise comparison of the criteria identified to prioritise the pest list, (iii) assigning a value to each criterion, based on the importance of the problem, (iv) calculating weightage for each criterion, (v) employing weightage of each criterion to calculate the value for each pest and (vi) ranking of the pest list identified, based on the overall weightage derived. In the current study, a list of seven criteria was developed as a strategy to reduce subjectivity and to keep experts focused on distinct issues. These were selected, based on its relevance to agriculture in the Caribbean region (Table 2):

**Table 2.** Caption text.

Criteria	Rationale for selection of the criteria
<b>1. Food Security</b>	Food security could be compromised in the Small Island Developing States when the crops are affected by the quarantine pests of importance and could result in civil unrest amongst the population in case of food insecurity. The region has been already toiling with a high food import bill of US\$13.76 billion from 2018–2020.
<b>2. Human Health</b>	The zoonotic nature of invasive plant pests of quarantine importance has been taken into consideration as it could potentially harm human health affecting the productive working population and collapsing the public health system. In addition, the nature of toxins produced by the quarantine pests of importance including mycotoxins were taken into consideration for listing this criterion in the list.
<b>3. Crop Health</b>	Healthy crop is critical for feeding the population with safe and nutritious food. When a pest of quarantine importance affects the crop health, it could result in demanding the application of highly hazardous pesticides which in-turn affect the environmental health and animal health.
<b>4. Aesthetic Value / Ecosystem Health</b>	As the Small Island Developing States highly depends on eco-tourism, the quarantine pests affecting the ecosystem health could hamper the economy generated from the tourism. For example, a pest of quarantine importance in coconut and palm trees could potentially destroy the ecosystem in the beaches/seashore. Additionally, when pests of horticultural or ecosystem-associated crops enter the region, management using pesticides would be a determinant to the ecosystem health.
<b>5. Production costs</b>	When quarantine pests of importance affect the crop, the costs incurred in eradication and or prevention of spread of a pest within the island or to the neighbouring island cultivating the same crop and management of a pest, are considered as critical in the Small Island Developing States as all crop production inputs are imported to the region.
<b>6. Foreign Trade / Exports</b>	Since the region has rich diversity and unique environment to produce high value crops that fetch good price at the international market through foreign trade, the entry of quarantine pests of such high-value crops (cocoa, spices, hot pepper) could destroy the industry. Hence, the quarantine pest and its impact on foreign trade was included in the selection criteria to prioritise the pest list.
<b>7. Public Costs</b>	When quarantine pests affecting the high-value crops or tourism industry, the governments would have to put forth the measures at community level supporting the farmers/producers through incentives, supply of chemical inputs etc. In this case, the spending of the public costs would be determined by the nature of quarantine pests. Hence, the reason for inclusion of the criterion in selection of pest list.

Since each country is unique, the criteria in one country could be more important than in another. Each criterion was, therefore, assigned a set of weights. A pair-wise comparison matrix was developed and each criterion was weighted relevant to the other. This process provided the judgements required to develop the matrices. Each criterion was compared with another criterion using a rating scale from 1 to 9 and weightage was developed as presented in Tables 3, 4 and 5 and Fig. 1. The process of identifying a prioritised pest list for the Caribbean using AHP was conducted by the Technical Committee in Tobago in 2014 and in Trinidad in 2018. The process was intensive and systematic with face-to-face sessions.

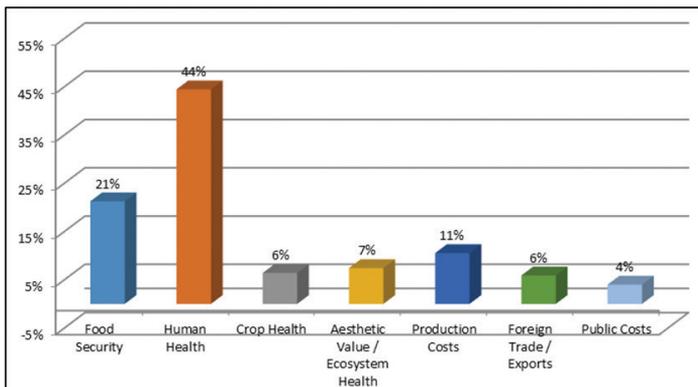
**Table 3.** Scale of importance for pairwise comparison of criteria set for prioritising pest list.

Intensity of importance	Definition	Explanation
1	Equal importance	Two elements contribute equally to the objective
3	Moderate importance	Experience and judgement slightly favour one element over another
5	Strong importance	Experience and judgement moderately favour one element over another
7	Very strong importance	One element is favoured very strongly over another, its dominance is demonstrated in practice
9	Extreme importance	The evidence favouring one element over another is of the highest possible order of affirmation

2, 4, 6, 8 can be used to express intermediate values. 1.1, 1.2 etc. for elements that are very close in importance.

**Table 4.** Pairwise comparison and ranking of the criterion identified for prioritising the pest list.

Matrix	Food Security	Human Health	Crop Health	Aesthetic Value / Ecosystem Health	Production Costs	Foreign Trade / Exports	Public Costs
Food Security	1	1/5	5	7	1	5	5
Human Health	5	1	5	7	7	5	5
Crop Health	1/5	1/5	1	5	1/3	1/5	3
Aesthetic Value / Ecosystem Health	1/7	1/7	1/5	1	3	3	3
Production Costs	1	1/7	3	1/3	1	3	3
Foreign Trade / Exports	1/5	1/5	5	1/3	1/3	1	1
Public Costs	1/5	1/5	1/3	1/3	1/3	1	1



**Figure 1.** Percentage of weight assigned to criteria, based on the importance of problem.

**Table 5.** Developing weightage to the criteria, based on the significance in relation to occurrence of pest.

Criterion	Mean value of comparison	Weight
Food Security	2.0914	0.2126
Human Health	4.3739	0.4446
Crop Health	0.6314	0.0642
Aesthetic Value / Ecosystem Health	0.7297	0.0742
Production Costs	1.0366	0.1054
Foreign Trade / Exports	0.5805	0.0590
Public Costs	0.3943	0.0401
<b>TOTAL</b>	<b>9.8378</b>	<b>1</b>

## Results

### Priority quarantine pest list identified in 2014 using DT and AHP

The use of the Delphi Technique by the experts has resulted in the determination of 15 regional priority pests for the Caribbean (Table 6). Five pests (Lethal yellowing in coconut; *Cyperus rotundus*, *Parthenium hysterophorus*: Giant African Snail, Black sigatoka leaf spot) that were reported as regulated quarantine pests in some of the islands were removed from the list of 15 before employing the AHP model. The Carambola Fruit Fly, Black Sigatoka leaf-spot and *Fusarium* Tropical Race 4 pathogens were listed as pests of quarantine importance due to their possible impacts on food security. Red Palm Weevil was included in the list, based on their impact on ecotourism as a result of the pest capacity to devastate the palm plants in those islands with large tourism industries. The Mediterranean Fruit Fly,

**Table 6.** Pests of quarantine importance for the Caribbean in 2014 using the Delphi Technique.

Subject Matter Specialist	Top 15 Pest of importance to the region	Criteria used to determine the pest being in the top 10 for the region
Entomology	Red Palm Weevil ( <i>Rhynchophorus ferrugineus</i> )	Aesthetic / Food Security / The Plant Propagative Material
	Mediterranean fruit fly ( <i>Ceratitis capitata</i> )	Production cost and domestic trade implications
	Carambola fruit fly ( <i>Bactrocera carambolae</i> )	Food security /Economic impact
	Cassava mite ( <i>Mononychellus tanajoa</i> )	Food security
	Tomato Leaf miner ( <i>Tuta absoluta</i> )	Food security
Fungi	Fusarium wilt in Banana ( <i>Fusarium oxysporum</i> f.sp. <i>cubense</i> TR4)	Food security/ Economic Impact
	Frosty pod rot in Cacao ( <i>Moniliophthora roveri</i> )	Trade implications
	Black Sigatoka leaf spot in Banana ( <i>Mycosphaerella fijiensis</i> )	Food security/ Trade implications / Natural Spread Pathway
Viruses	Citrus leprosis virus	Trade implications
Bacteria / Phytoplasma	Bacterial wilt in banana ( <i>Ralstonia solanacearum</i> )	Production cost and domestic trade implications
	Citrus canker ( <i>Xanthomonas citri</i> subsp. <i>citri</i> )	Trade implications
	Lethal yellowing in coconut ( <i>Candidatus Phytoplasma</i> )	Economic and environmental impact (aesthetic value)
Weeds	Nut grass ( <i>Cyperus rotundus</i> )	Food Security / Economic Impact / Human Movement Pathway
	White top ( <i>Parthenium hysterophorus</i> )	IAS / Food security/ Public cost
Molluscs	Giant African Snail ( <i>Achatina fulica</i> )	Human Health / Public cost / Hitch hiking Pest

Frosty Pod Rot, Citrus Leprosis Virus and Moko Wilt pathogens topped the list for domestic and international trade implications. The pests identified using DT, however, did not prioritise one pest over another, but rather only listed the top 10 pests. It was, therefore, difficult for the national authorities to allocate resources to mitigate risk through surveillance and the development of emergency action plan for management. This was overcome using the AHP process, which dexterously used the weightage for each criterion to rank the pest.

In the present study, the AHP model assigned a higher weight to the human health criterion (44%), followed by food security (21%). The lowest weight was assigned to the public costs criterion at 4%. The exercise conducted in 2014 ranked the Mediterranean Fruit Fly (*Ceratitidis capitata*) as a pest of high-risk importance, followed by the Carambola Fruit Fly (*Bactrocera carambolae*). The least importance was given to the Cassava Mite and Citrus Leprosis Virus. *Fusarium* Wilt TR4 that received global attention during this assessment period found a place in the top 5 list (Table 7).

### Priority quarantine pest list identified in 2018 using DT and AHP

The exercise conducted in 2018 employing DT and AHP methods respectively identified and prioritised the top 10 regional quarantine pests (Table 8). In 2018, the Mediterranean Fruit Fly (insect) was ranked as a pest of high risk to the region followed by *Fusarium* TR4 (fungus) and Tomato Leaf Miner (insect). The Bacterial Wilt, Frosty Pod Rot and Lethal Yellowing were assigned a moderate score by the AHP model. Citrus Canker and Leprosis, Fiji Disease in sugar-cane and Bacterial Panicle Blight in rice were rated low in the model (Table 8).

## Discussion

The present study was an attempt to hone the process of developing a regional priority pest list using a multi-criteria decision-making approach. The Delphi Technique was useful in the current study, based on the discussion, peer review, consultation and opinion of

**Table 7.** Prioritised quarantine pests identified for the Caribbean using the Analytic Hierarchy Process in 2014.

Pest list derived from Delphi Technique	Weighted Score	Final AHP Ranking of Invasive Alien Species
Pest #1: <i>Bactrocera carambolae</i> (Carambola Fruit Fly)	0.153	2
Pest #2: <i>Fusarium oxysporum</i> f.sp. <i>cubense</i> Race 4 (Banana Fusarium Wilt)	0.130	4
Pest #3: <i>Moniliophthora roreri</i> (Cocoa Frosty Pod)	0.065	7
Pest #4: <i>Tuta absoluta</i> (Tomato Leaf Miner)	0.126	5
Pest #5: <i>Ceratitidis capitata</i> (Mediterranean Fruit Fly)	0.166	1
Pest #6: Citrus leprosis virus (Leprosis of citrus)	0.048	9
Pest #7: Citrus canker ( <i>Xanthomonas citri</i> subsp. <i>citri</i> )	0.053	8
Pest #8: <i>Mononychellus tanajoa</i> (Cassava Mite)	0.045	10
Pest #9: <i>Ralstonia solanacearum</i> (Moko Disease Race 2)	0.138	3
Pest #10: <i>Rhynchophorus ferrugineus</i> (Red Palm Weevil)	0.076	6

**Table 8.** Prioritised quarantine pests identified for the Caribbean using the Analytic Hierarchy Process in 2018.

	<b>Pest list derived from Delphi Technique</b>	<b>Weighted Score</b>	<b>Final AHP Ranking of Invasive Alien Species</b>
A	Pest #1: <i>Burkholderia glumae</i> (Rice Bacterial Panicle Blight)	0.064	10
B	Pest #2: Fiji disease virus (Fiji Disease in sugarcane)	0.066	9
C	Pest #3: <i>Fusarium oxysporum</i> f.sp. <i>cubense</i> (Fusarium Tropical Race 4)	0.125	2
D	Pest #4: <i>Ceratitis capitata</i> (Mediterranean Fruit Fly)	0.221	1
E	Pest #5: <i>Ralstonia solanacearum</i> (Moko wilt in banana)	0.100	4
F	Pest #6: <i>Candidatus Phytoplasma</i> (Lethal yellowing in coconut)	0.086	6
G	Pest #7: <i>Tuta absoluta</i> (Leaf miner in tomato)	0.113	3
H	Pest #8: Citrus Leprosis Virus (Leprosis of Citrus)	0.068	8
I	Pest #9: <i>Moniliophthora roreri</i> (Cocoa Frosty Pod)	0.088	5
J	Pest #10: <i>Xanthomonas citri</i> subsp. <i>citri</i> (Citrus canker)	0.069	7

the experts. This was evident from the process, during which the regional technical committee initially attempted to employ the Point Score Analysis in prioritising the pest list, based on the survey with less rigour and lack of scientific evidence (data not presented).

While comparing the DT with the AHP model, the latter was seen as a structured technique for organising and analysing complex decisions. It has been used around the world in a wide variety of strategic decision-making situations, in areas such as border disputes, government, business, industry, healthcare and education. Given the complexity of some problems and the number of factors that should be simultaneously considered to derive the best possible outcome, the AHP boasts of going beyond prescribing a “correct” decision; rather, it can help decision-makers find an option that best suits their goal and their understanding of the problem. It provides a comprehensive and rational framework for structuring a decision problem, for representing and quantifying its elements, for relating those elements to overall goals and for evaluating alternative solutions (Wan et al. 2005; Szabo et al. 2021). The AHP Prioritised Pest List criteria set a strategic objective to identify high-risk quarantine pests for early detection.

The prioritisation process gave way to the first Regional Priority Pest List being completed in 2014 identifying the top 10 pests of regional priority and then once again in 2018. The Pest Prioritisation exercises were seen to be both useful and instructive for the region as the lists identified several key pests that were subsequently detected in the region: Mediterranean Fruit Fly (*Ceratitis capitata*) in the Dominican Republic in 2015 (Zavala-López et al. 2021), Frosty Pod Rot in Jamaica in 2016 (Ministry of Agriculture, Fisheries and Mining, Jamaica 2021) and *Tuta absoluta* in Haiti in May 2019 (Verheggen and Fontus 2019).

The outbreak of Mediterranean Fruit Fly was reported in the Dominican Republic in March 2015, causing an export revenue loss of US\$ 40 million within 10 months of outbreak, risking 30,000 jobs (Zavala-López et al. 2021). The rapid action taken by the government, in collaboration with the FAO, the International Atomic Energy Agency (IAEA), the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS), Organismo Internacional Regional de Sanidad Agropecuaria (OIRSA) and the Inter-American Institute for Cooperation on Agriculture

(IICA), successfully eradicated the fruit fly in January 2017 through application of the sterile insect technique and integrated pest management practices. This has protected the horticulture industry of neighbouring countries in the Caribbean, Mexico and United States, circumventing significant economic losses (Zavala-López et al. 2021). It is significant to note that this pest was ranked in the top priority quarantine pest list developed in 2014 using the multi-criteria decision approach.

Similarly, various reports had been received in Jamaica that farmers were losing 40–90% of their production due to frosty pod rot disease. It was estimated to destroy anywhere between 80–100% of the entire production in the island. The first case of the frosty pod rot disease was reported to the Ministry of Agriculture in August 2016, at which point it was discovered that the parish of Clarendon was heavily infested. In a matter of approximately 4–5 months, the disease was discovered to be in the neighbouring parishes of St. Catherine, St. Andrew and St. Mary. It was suspected that the pathway of entry of the frosty pod rot disease would have been through human transmission. Based on molecular analysis, it was discovered that the strain of the frosty pod rot disease in Jamaica is similar to that in Columbia. The prioritisation of pests has assisted the region to develop strategies in advance for the management of the pests where they anticipate an entry and spread into the territory. In this case, the strategies employed in Jamaica included: (i) a delimiting survey to identify how far the disease spread, (ii) training and sensitisation of farmers and other stakeholders of the disease, (iii) creation of a buffer zone that eliminated all the conditions that the disease requires to survive and (iv) training of officers in management practices. Similarly, Huanglongbing or citrus greening disease prioritised for the Caribbean region, though identified in Jamaica earlier, was later detected in Trinidad in 2017, leading to the destruction of 200,000 citrus trees as per the Ministry of Agriculture, Trinidad and Tobago. Tomato leaf miner, ranked in the top 10 list from the 2014 and 2018 exercises, was subsequently reported in 2019 and 2021, respectively, in Haiti and Trinidad. This could pose a potential threat for dissemination to the Dominican Republic and North American countries. This has clearly demonstrated the relevance and usefulness of the priority exercise employed in the development of the regional priority pest list.

Most importantly, the use of pest prioritisation techniques has been highly beneficial to the SIDS that are characterised with poor capacity in allocating resources. The prioritisation process can guide SIDS to precisely direct its resources for the prevention and management of the quarantine pests. In addition, the employment of pest prioritisation techniques will be highly useful in determining the pathways and alerting the inspection processes at air and water borders in SIDS which are highly vulnerable to the entry of invasive pests through tourism-related activities. The prioritisation process has furthermore strengthened the local knowledge throughout the region on quarantine pests of importance. The methods used in the study have flexibility to include or exclude the criteria relevant to the changing and demanding needs of region. This model could be employed elsewhere to prioritise the national or regional pest list, based on the criterion relevant to the region. Though the AHP model permitted the ranking of the pest list, based on the relative importance of the criteria proposed, some

challenges still remain due to the dependency of the model on the provision of strong scientific evidence and receipt of an unbiased list of pests. Similar to techniques, such as the Horizon Scanning method (Kendig et al. 2022) which employs arrival, establishment and impact of potential pests, the current study systematically combined and gave equal weightage to invasiveness, spread, establishment and impact to generate the pest list and then used the criteria to rank the pest list. In this context, exploring and comparison of risk-based methods, such as Horizon Scanning and the Pest Assessment and Prioritisation Process (OPEP) models with the current technique could provide further validation and reliability of the models in making multi-criteria decisions in fields like agriculture where many factors need to be considered. In addition, continuous monitoring and recording of the impact of these predictions after prioritising the pest list could provide further insights in improving these models.

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## Supplementary material I

### **AHP model developed for priotization of quarantine plant pests in the Caribbean**

Authors: Govind Seepersad, Camelia Ram

Data type: xlsx

Explanation note: The Model can be modified and used for prioritizing plant pests.

Copyright notice: This dataset is made available under the Open Database License (<http://opendatacommons.org/licenses/odbl/1.0/>). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

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# Running rampant: the alien ants (Hymenoptera, Formicidae) of Cyprus

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## Abstract

Biological invasions are considered a major driver of biodiversity loss, particularly on islands. Invasive alien ants can often have severe consequences on native biodiversity. Here, we review published and new information on alien ant species found on the Mediterranean island of Cyprus, a biodiversity hotspot. Our checklist of alien ants of Cyprus includes a total of 17 species, of which nine are reported from Cyprus for the first time (\*): *Camponotus* cf. *vitiosus* Smith, *Cardiocondyla mauritanica* Forel, 1890, *Cardiocondyla obscurior* Wheeler, W.M., 1929\*, *Hypoponera punctatissima* (Roger, 1859)\*, *Monomorium bicolor* Emery, 1877, *Nylanderia jaegerskioeldi* (Mayr, 1904), *Paratrechina longicornis* (Latreille, 1802), *Pheidole fadli* Sharaf, 2007\*, *Pheidole indica* Mayr, 1879, *Solenopsis* sp. (thief ant)\*, *Tetramorium bicarinatum* (Nylander, 1846)\*, *Tetramorium caldarium* (Roger, 1857)\*, *Tetramorium immigrans* Santschi, 1927\*, *Tetramorium lanuginosum*

Mayr, 1870\*, *Trichomyrmex destructor* (Jerdon, 1851), *Trichomyrmex mayri* (Forel, 1902)\*, and *Wasmannia auropunctata* (Roger, 1863). We did not include three previously reported alien species for which we could not find supporting specimens [*Monomorium pharaonis* (Linnaeus, 1758), *Nylanderia vividula* (Nylander, 1846), *Solenopsis geminata* (Fabricius, 1804)], one based on a previous misidentification [*Cardiocondyla nuda* (Mayr, 1866)], and two species now considered native to Cyprus [*Hypoponera eduardi* (Forel, 1894), *Monomorium subopacum* (F. Smith, 1858)]. Literature records, specimens from field surveys and museum collections, the geographic origin of species, occupied habitats in Cyprus, and notes on invasiveness (spread and impact) are presented for each species. An identification key to distinguish alien from native ant species in Cyprus is provided, including widespread alien ants not yet known from Cyprus in order to support early detection, monitoring, and management efforts.

### Keywords

biological invasions, checklist, first records, identification key, invasive alien species, social insects, tramp species

## Introduction

Biological invasions are considered a major driver of global biodiversity loss, with profound impacts on the extinction risk and evolutionary histories of island species (Mooney and Cleland 2001; Reaser et al. 2007; Butchart et al. 2010; Russell et al. 2017; Leclerc et al. 2018; IPBES 2019). Of 520 ant species found transported outside their native range globally, almost half of them have managed to establish viable, self-sustaining populations in their invaded range, although only relatively few have been assessed as harmful (Wong et al. 2023). A recent study assessing socio-economic and environmental impacts of alien ants, documented 31 species as highly invasive (Gruber et al. 2022). Alien ants have been found to have adverse environmental impacts on native flora and fauna, largely through competition and predation, and in some cases lead to the extinction of native species. Additionally, alien ants have serious socio-economic impacts as household pests, and threaten human and animal health as both vectors of pathogens and through their venomous stings which may cause allergic reactions (Angulo et al. 2022; Gruber et al. 2022).

The Mediterranean Basin is a global biodiversity hotspot with a plethora of endemic species in need of conservation (Myers et al. 2000). Situated at the crossroad between three continents, Cyprus constitutes the 3<sup>rd</sup> largest Mediterranean island covering an area of 9,251 km<sup>2</sup>. The island is a centre of endemism for Mediterranean plants hosting 146 endemic species and subspecies (8.85% endemism rate) (Hand et al. 2011, 2019; Cheikh Albassatneh et al. 2021) and is also considered a centre of endemism for mammals (Hadjisterkotis and Masala 1995) as well as European and Middle Eastern birds (Bibby et al. 1992). Regarding its insect biota, recent comprehensive studies on the wild bees of Cyprus indicate the presence of 21 endemic species of Anthophila (5.7% endemism rate) (Varnava et al. 2020). Data on other hymenopteran taxa remain unpublished or largely unknown.

The myrmecofauna of Cyprus is considered relatively understudied, with published reports of 65 native and nine alien species (Janicki et al. 2016; Guénard et al.

2017); constituting fewer species than the neighbouring island of Crete, an island of similar area, where 93 native and 10 alien species have been collected (Salata et al. 2020; Lapeva-Gjonova et al. in prep). Nonetheless, a number of endemic species and subspecies have been described from Cyprus, including *Cataglyphis aphrodite* Salata, Demetriou, Georgiadis & Borowiec, 2023, *Cataglyphis chionistrae* Salata, Demetriou, Georgiadis & Borowiec, 2023 (Salata et al. 2023a), *Crematogaster cypria* Santschi, 1930 (Salata and Borowiec 2015b), *Lasius cyperus* Seifert, 2020 (Seifert 2020), and *Oxyopomyrmex pygmalioni* Salata & Borowiec, 2015 (Salata and Borowiec 2015a), as well as subspecies such as *Crematogaster inermis aphrodite* Santschi, 1937, *Solenopsis fugax cypridis* Santschi, 1934 and *Temnothorax bulgaricus cypridis* (Santschi, 1930) (Santschi 1930, 1934, 1937). In addition, several more endemic taxa are currently being described (Salata, Demetriou, Georgiadis and Borowiec, unpubl. data).

The first checklist of the alien ants of Cyprus (Salata et al. 2019) and a recent review on the island's alien insect-fauna (Demetriou et al. 2023a) both included nine species of ants, though these checklists differed in four species. Here, we review published and new information on alien ants found on the Mediterranean island of Cyprus. We perform a focused study on the alien ant fauna of Cyprus, both to provide a more comprehensive list, and reconcile existing incongruences in the literature regarding ant species presence on the island. Considering the adverse environmental and socio-economic impacts of alien ants, an updated, annotated checklist is provided alongside an identification key to distinguish alien from native ant species of Cyprus. The presence of alien ants in the NATURA 2000 network and their land-cover usage are analysed and discussed.

## Materials and methods

### Data collection and specimen identification

A literature review was carried out to collate all available records of alien ants reported from Cyprus including species catalogued in the Global Ant Biodiversity Informatics (GABI) database (Janicki et al. 2016; Guénard et al. 2017) and scientific literature (Emery 1909, 1910; Georghiou 1977; Georgiadis et al. 2017; Salata et al. 2019; Demetriou et al. 2022, 2023a). In addition, specimens collected in the field by the authors on the island during 2012, 2021 and 2022, deposited in the collections of L. Borowiec and S. Salata (Department of Biodiversity and Evolutionary Taxonomy, University of Wrocław, Poland – DBET), Ch. Georgiadis (Museum of Zoology of the University of Athens, Greece – ZMUA), J. Wetterer (Smithsonian National Museum of Natural History, Washington, USA) and J. Demetriou (pers. collection) as well as material in the Museum of Natural History of the University of Wrocław, Poland (MNHW) and Natural History Museum of Crete (NHMC) were examined (Suppl. material 1).

The identification of the ants was performed by comparing the specimens with type material deposited in museum collections such as that of MNHW alongside regional

works on European ants and recent published revisions for genera and species complexes/groups (Agosti and Collingwood 1987; Seifert 2003, 2018, 2020; Czechowski et al. 2012; Seifert et al. 2017; Wagner et al. 2017).

## Establishment status

The establishment status of alien ants was catalogued as Established i.e. “non-native species records with established populations in the wild” or Indoors introduced i.e. “non-native species records without established populations in the wild (e.g. in buildings, greenhouses, airports, quarantine surveys)” noting that within GABI the equivalent categories are given as exotic (= established) and indoors introduced (Janicki et al. 2016; Guénard et al. 2017). Lastly, the establishment status of data-deficient species is given as “Unknown”.

The native origin of species was decided based on GABI accessed through the antmaps.org website (Janicki et al. 2016; Guénard et al. 2017) and available scientific literature on the native range of species. Each species was assigned to one or more biogeographic realms sensu Holt et al. (2013). Criteria in Essl et al. (2018) were also applied to determine whether some species could be regarded as alien or native.

## Identification key

A dichotomous identification key to distinguish alien from native ant species inhabiting Cyprus was constructed using available scientific literature and specimens (Seifert 2003; Sharaf et al. 2016; Salata et al. 2020; Gotzek and Martinez 2021; Borowiec and Salata 2022). Additionally, several alien and invasive alien species included in the 100 of the world’s worst invasive alien species (*Anoplolepis gracilipes*, *Linepithema humile*, *Pheidole megacephala*, *Solenopsis invicta* and *Wasmannia auropunctata*) (GISD 2023), alien invasive species of Union Concern (*Solenopsis geminata*, *S. invicta*, *S. richteri* and *W. auropunctata*) (EU 2022/1203), species listed in a horizon scanning exercise (Peyton et al. 2019) (*L. humile* and *W. auropunctata*), alien ant species inhabiting the Eastern Mediterranean (Janicki et al. 2016; Guénard et al. 2017) as well as international and regional lists of widespread alien ants (Vonshak and Ionescu-Hirsch 2009; Wetterer 2009a, 2014a, b; Pospischil 2011; Borowiec 2014; Schifani 2019; Kiran and Karaman 2020; Salata et al. 2020; Pawluk et al. 2022a, b; Demetriou et al. 2023a) were added to the identification key to facilitate early detection, monitoring and management efforts across Cyprus.

## Spread and invaded habitats

The distribution of alien ant species within Cyprus was mapped. A total of 281 geo-referenced observations (Suppl. material 1) were pooled in QGIS Version 3.18.2 free and open source Geographic Information System (<https://qgis.org/en/site/>) and were assigned to their respective land cover and presence within the NATURA2000 network.

Boundaries and habitat types were based on the European layers of Corine Land Cover (CLC) project version CLC2018 and NATURA2000 sites, downloaded from Copernicus Land Monitoring Service and the European Environmental Agency, respectively. The occurrence of species within the boundaries of the protected RAMSAR site of Akrotiri (<https://rsis.ramsar.org/RISapp/files/1375/pictures/GB1375map.pdf>) (RAMSAR site code: 1375), was also documented. In addition to these distribution maps, information on occupied habitats are given, providing additional information alongside the broad CLC types. Therefore, with reference to the Copernicus Land Monitoring Service, records of ants within the following land-cover types were mapped: artificial surfaces, agricultural areas, forest and semi-natural areas, wetlands and water bodies as well as their respective sub-categories (<https://land.copernicus.eu/user-corner/technical-library/corine-land-cover-nomenclature-guidelines/html>). The area of occupancy (AOO) and extent of occurrence (EOO) of species were calculated through GeoCAT in a  $2 \times 2$  km<sup>2</sup> grid, following guidelines of the International Union for the Conservation of Nature (IUCN) for the assessment of extinction risk of taxa (IUCN 2001; Bachman et al. 2011).

### Specimen photography

Photographs of specimens, unless stated otherwise, were taken by Prof. L. Borowiec using Nikon SMZ18 and Nikon SMZ 1500 stereomicroscopes, Nikon D5200 camera and Helicon Focus software. Locality data for each photographed specimen are provided in the figure titles.

### Results

The updated checklist of alien ants of Cyprus currently comprises of 17 species (Table 1). Six species previously included on checklists were removed. In particular, records of *Cardiocondyla nuda* (Mayr, 1866), *Monomorium pharaonis* (Linnaeus, 1758), *Nylanderia vividula* (Nylander, 1846) and *Solenopsis geminata* (Fabricius, 1804) are considered as dubious, while *Hypoponera eduardi* (Forel, 1894) and *Monomorium subopacum* (Smith, F., 1858) are currently considered as native to the island. Furthermore, eleven species are added, including the recently detected invasive alien *Wasmannia auropunctata* (Roger, 1863) (Demetriou et al. 2022), *Camponotus* cf. *vitiosus* (Salata et al. 2023b) and newly presented records of *Pheidole fadli* Sharaf, 2007, *C. obscurior* Wheeler, W.M., 1929, *Tetramorium bicarinatum* (Nylander, 1846), *T. caldarium* (Roger, 1857), *T. immigrans* Santschi, 1927, *T. lanuginosum* Mayr, 1870, *Solenopsis* sp\_CYP139, *T. mayri* (Forel, 1902), and *H. punctatissima* (Roger, 1859) (Tables 1, 2).

The five species with the highest AOO in decreasing order are *M. bicolor*, *P. indica*, *N. jaegerskioeldi*, *P. longicornis* and *W. auropunctata*, while regarding their EOO this order is: *T. bicarinatum*, *M. bicolor*, *P. indica*, *P. longicornis* and *N. jaegerskioeldi*, in decreasing order (Table 2). The majority of alien species have been collected in the island's lowlands, below the altitude of 500 m. Nevertheless, *M. bicolor* and *T. mayri*

**Table 1.** List of alien ants of Cyprus and accompanying notes including previous lists, (1) Salata et al. (2019), (2) Demetriou et al. (2023a), and updates from (3) Present study.

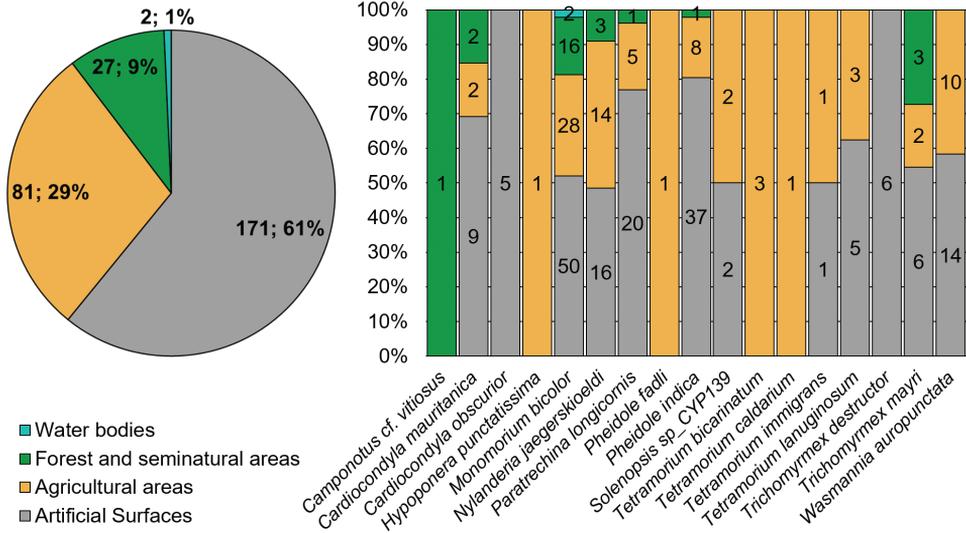
Subfamily	Tribe	Species	1	2	3	Notes
Formicinae	Camponotini	<i>Camponotus</i> cf. <i>vitiosus</i>			X	
	Lasiini	<i>Nylanderia jaegerskioeldi</i>	X	X	X	
Myrmicinae	Attini	<i>Paratrechina longicornis</i>	X	X	X	
		<i>Pheidole fadli</i>			X	New addition – new record
		<i>Pheidole indica</i>	X	X	X	
	Crematogastrini	<i>Wasmannia auropunctata</i>			X	
		<i>Cardiocondyla mauritanica</i>	X	X	X	
		<i>Cardiocondyla nuda</i>		X		Removed as dubious
		<i>Cardiocondyla obscurior</i>			X	New addition – new record
		<i>Tetramorium bicarinatum</i>			X	New addition – new record
		<i>Tetramorium caldarium</i>			X	New addition – new record
		<i>Tetramorium immigrans</i>			X	New addition – new record
		<i>Tetramorium lanuginosum</i>			X	New addition – new record
	Solenopsidini	<i>Monomorium bicolor</i>	X	X	X	
		<i>Monomorium pharaonis</i>	X	X		Removed as dubious
		<i>Monomorium subopacum</i>	X			Removed as native
		<i>Solenopsis geminata</i>	X	X		Removed as dubious
<i>Solenopsis</i> sp_CYP139				X	New addition – new record	
<i>Trichomyrmex destructor</i>			X	X		
Ponerinae	Ponerini	<i>Trichomyrmex mayri</i>			X	New addition – new record
		<i>Hypoponera eduardi</i>	X			Removed as native
		<i>Hypoponera punctatissima</i>			X	New addition – new record
<b>Total alien species</b>			<b>9</b>	<b>9</b>	<b>17</b>	

have reached altitudes of 550 and 600 metres, respectively, and *C.* cf. *vitiosus* has been collected in the Cedar Valley protected area (CY2000006) at an altitude of approx. 1200 m. A total of seven alien species were collected from 20 protected areas within the NATURA 2000 and RAMSAR network. *Monomorium bicolor* was found in 15 protected sites, followed by *P. longicornis* and *T. mayri* each in three. The Akamas Peninsula (sites CY4000010 and CY4000023) was the protected area with the highest number of alien ants (hosting four alien species), followed by the Akrotiri Peninsula RAMSAR site with three alien species. The sites Koili – Mavrokolympos (CY4000008), Faros Kato Paphou (CY4000013), Alykes Larnakas (CY6000002) and Periochi Kosiis – Pallourokampou (CY6000009) each hosted two alien species, with the remaining protected areas having only one alien species (Suppl. material 2). Only three alien species have been reported from Cyprus during the 20<sup>th</sup> century. The remaining 14 species were recorded within the last decade (2012–2022).

According to the CLC analysis, 90% of georeferenced records of alien ants have been collected from anthropogenic habitats (Fig. 1). These include artificial surfaces (61%) and agricultural areas (29%), with further information provided in Suppl. material 1. Only 9% of records were associated with semi-natural and natural areas, with two records (1%) corresponding to inland water bodies, specifically ants collected close to water reservoirs.

**Table 2.** Spatiotemporal characteristics of alien ants species in Cyprus, including their area of occupancy (AOO) and extent of occurrence (EOO) in a  $2 \times 2$  km<sup>2</sup> grid, altitudinal range in metres (rounded off to the nearest 50s), number and code(s) of occupied protected areas as well as the year of first official published record and associated reference.

No.	Taxonomy	Spread (km <sup>2</sup> )		Altitude range (m)	Protected areas		First official published record	
	Species	AOO	EOO		No.	Code(s)	Detection year	Reference
1	<i>Camponotus</i> cf. <i>vitiosus</i>	N/A	N/A	1200	2	CY2000006 CY2000016	2012	Salata et al. 2023b
2	<i>Nylanderia jaegerskioeldi</i>	80	4,728	0–400	5	CY4000008 CY4000010 CY4000023 CY6000009 RAMSAR1375	1910	Emery (1910)
3	<i>Paratrechina longicornis</i>	72	4,915	0–150	3	CY4000010 CY4000023 CY6000002	2012	Salata et al. (2019)
4	<i>Pheidole fadli</i>	N/A	N/A	100	0	N/A	2022	present study
5	<i>Pheidole indica</i>	100	4,963	0–400	2	CY4000008 CY4000013	2012	Salata et al. (2019)
6	<i>Wasmannia auropunctata</i>	60	410	0–300	0	N/A	2022	Demetriou et al. (2022)
7	<i>Cardiocondyla mauritanica</i>	36	4,043	0–150	0	N/A	1909	Emery (1909)
8	<i>Cardiocondyla obscurior</i>	16	65	0–50	0	N/A	2012	present study
9	<i>Tetramorium bicarinatum</i>	12	9,878	0–100	0	N/A	2022	present study
10	<i>Tetramorium caldarium</i>	N/A	N/A	100	0	N/A	2022	present study
11	<i>Tetramorium immigrans</i>	8	N/A	0–100	0	N/A	2022	present study
12	<i>Tetramorium lanuginosum</i>	28	247	0–100	2	CY4000010 CY4000023	2013	present study
13	<i>Monomorium bicolor</i>	264	6,318	0–600	15	CY3000007 CY3000008 CY4000003 CY4000005 CY4000007 CY4000010 CY4000013 CY4000019 CY4000020 CY4000021 CY4000023 CY4000025 CY6000002 CY6000009 RAMSAR1375	2012	Salata and Borowiec (2015b)
14	<i>Solenopsis</i> sp_CYP139	16	1,185	0–100	0	N/A	2022	present study
15	<i>Trichomyrmex destructor</i>	16	159	0–100	0	N/A	1925	Georghiou (1977)
16	<i>Trichomyrmex mayri</i>	32	4,074	0–550	3	CY2000004 CY2000013 RAMSAR1375	2022	present study
17	<i>Hypoponera punctatissima</i>	N/A	N/A	100	0	N/A	2022	present study



**Figure 1.** Number of georeferenced records of alien ants and respective percentage within broad Corine Land Cover (CLC) types in Cyprus (A). Percentage and number of georeferenced records for each species of alien ant collected in Cyprus per broad CLC type (B).

## Commented checklist

### Alien ants of Cyprus

#### Formicinae

#### Formicini

#### *Camponotus cf. vitiosus*

Fig. 2

**Literature records.** (Salata et al. 2023b).

**Material examined.** Suppl. material 1.

**Origin.** Sino-Japanese biogeographic realm.

**Habitat details.** Collected once from Cedar valley (Paphos), in high altitude (1196 m) in natural pine and cedar forest (Salata et al. 2023b).

**Degree of establishment.** Unknown.

**Notes.** A species resembling *C. vitiosus* was first recorded for the Mediterranean Basin from Israel, as *Camponotus (Myrmamblys) sp. near vitiosus* Smith, F., 1874 (Ionescu-Hirsch 2009). Specimens were intercepted at the Ashdod Port “in bamboo from China” (Ionescu-Hirsch 2009), while no additional records of the species have been provided since. A worker and a queen of the species were collected from the Cedar valley in 2012, situated within the Troodos mountain range, and the overlapping protected NATURA 2000 sites CY2000006 and CY2000016. Follow-up field surveys in 2022 provided no further material. In its native range (China, Japan and Korea),



**Figure 2.** Habitus of minor worker of *Camponotus* cf. *vitiosus* (from Paphos, Cedar Valley) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

*C. vitiosus* has been collected both from urban and natural habitats in the lowlands and high mountains (Guénard and Dunn 2012; Kim and Lyu 2012; Park et al. 2014). The exact identity of this morphospecies will be further investigated with the help of molecular tools and sampling to retrieve any additional material.

## Lasiini

### *Nylanderia jaegerskioeldi* (Mayr, 1904)

Fig. 3

**Literature records.** Emery (1910); Georgiadis et al. (2017); Salata et al. (2019).

**Material examined.** Suppl. material 1.

**Origin.** Africa and Arabian Peninsula (Afrotropical and Saharo-Arabian biogeographic realms).

**Habitat details.** Common synanthropic species collected from urban habitats, plant nurseries, and households as well as from semi-natural and natural habitats such as a beach, stream valley, canyon, pastureland, and a river bank.

**Degree of establishment.** Established.

**Notes.** In Sicily aggressive behaviour towards *Pheidole pallidula* (Nylander, 1849) has been observed, although typically across the Mediterranean *N. jaegerskioeldi* is mostly collected from anthropogenic habitats (Obregón Romero and Reyes López 2012; Schifani and Alicata 2018; Reyes-López and Taheri 2018). In Cyprus, the species has been found indoors as a household pest, as well as in five natural protected areas including the Akrotiri marsh RAMSAR site. More research on its impacts on native ant communities is required.

### *Paratrechina longicornis* (Latreille, 1802)

Fig. 4

**Literature records.** Salata et al. (2019).

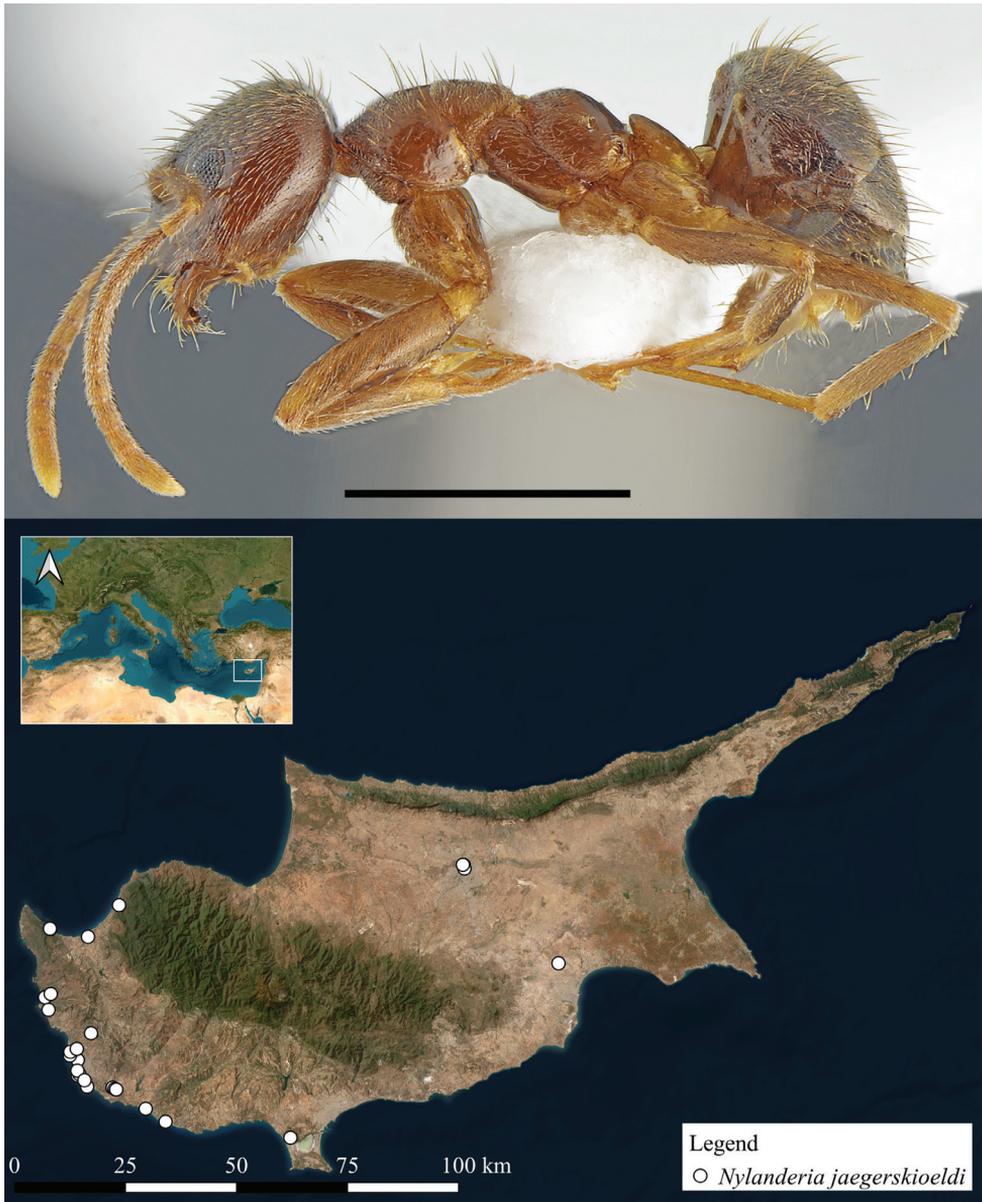
**Material examined.** Suppl. material 1.

**Origin.** Indian subcontinent (Tseng et al. 2022) (Indomalayan biogeographic realm).

**Habitat details.** Common species in urban areas (garden, parks, parking lots, roadsides) and disturbed semi-natural habitats (beach, dirt road, field, waterfront).

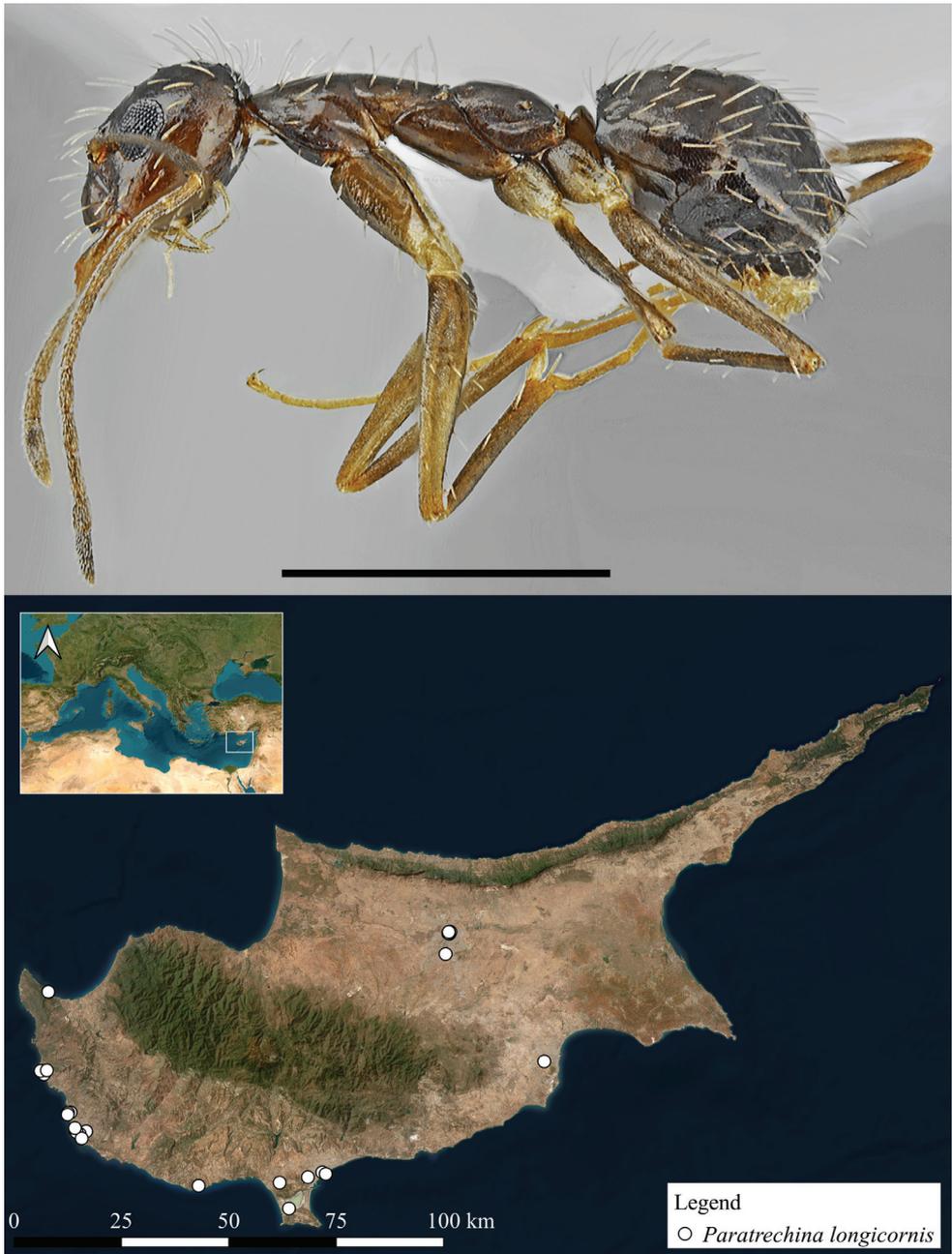
**Degree of establishment.** Established.

**Notes.** This synanthropic species is widespread across Cyprus and has been collected from a variety of habitats. In Greece, *P. longicornis* can be found in the Dodecanese, inhabiting disturbed habitats in the lowlands and invading households (Borowiec and Salata 2022). Nevertheless, the species has been found to co-occur with native species, showing no signs of aggression (Salata et al. 2019). In Cyprus, the species can be found in urban and semi-natural habitats with evident human intervention and no adverse impact on native ant species has been observed. Nevertheless, detailed studies on the



**Figure 3.** Habitus of *Nylanderia jaegerskioeldi* (Mayr, 1904) (specimen from Paphos, Baths of Aphrodite) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

impacts of *P. longicornis* on native biodiversity are necessary. *Paratrechina longicornis* is also known to tend honeydew producing Hemiptera such as aphids (Saddiqui et al. 2019). In Cyprus, it was collected from *Eucalyptus* foliage probably feeding on honeydew secreted from *Glycaspis brimblecombei* Moore, 1964. Such a behaviour could exacerbate the socio-economic impacts of the psyllid. Further research on the impacts



**Figure 4.** Habitus of *Paratrechina longicornis* (Latreille, 1802) (specimen from Paphos, Baths of Aphrodite) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

of the species as a household pest and its interactions with agricultural pests such as aphids or the alien cricket *Myrmecophilus americanus* Saussure, 1877 (Wetterer and Hugel 2014) could be prioritised.

**Myrmicinae**  
**Attini**

***Pheidole fadli* Sharaf, 2007**

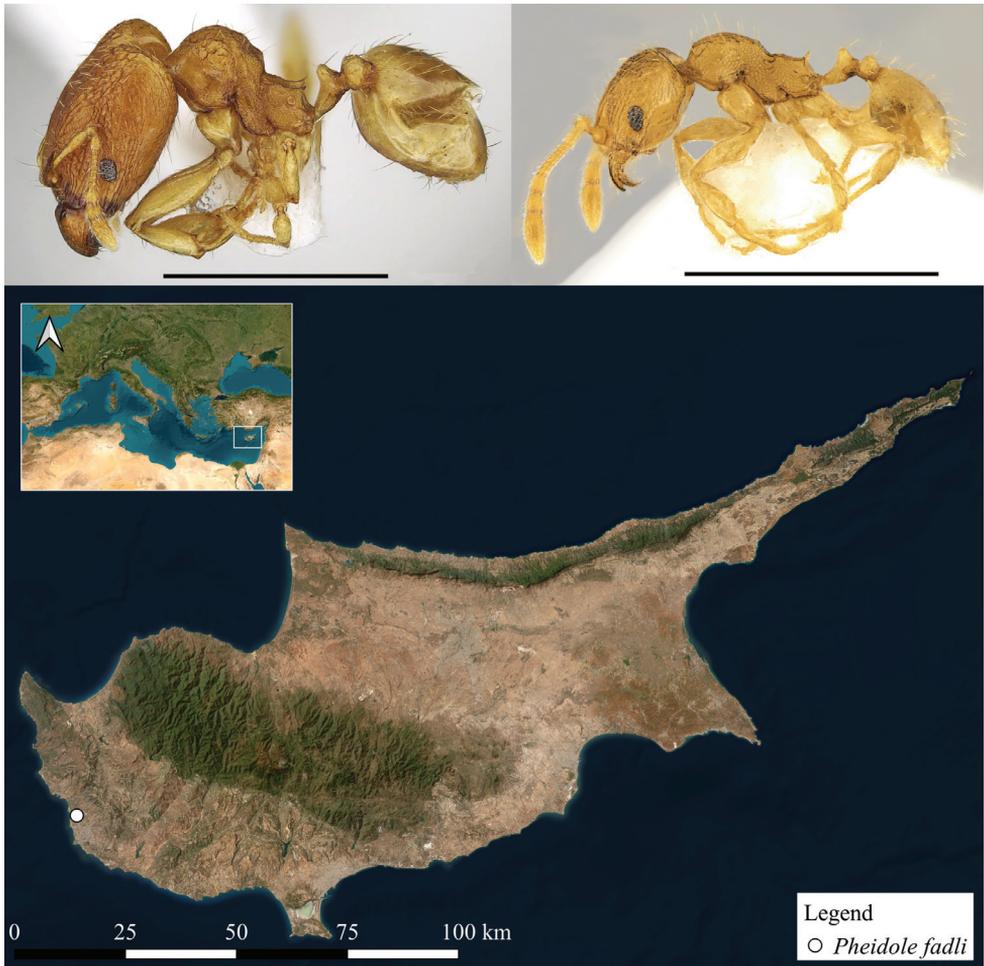
Fig. 5

**Literature records.** N/A.

**Material examined.** Suppl. material 1.

**Origin.** Egypt (Sino-Arabian biogeographic realm).

**Habitat details.** Only one specimen collected from a plant nursery in Paphos. Probably unintentionally introduced through the horticultural pathway in soil.



**Figure 5.** Habitus of *Pheidole fadli* Sharaf, 2007 major worker (left, from Egypt, Aswan, photographed by Michele Esposito, from [www.antweb.org](http://www.antweb.org), CASENT0919803) and minor worker (right, from Paphos, Lemba) in lateral view (scale bar: 1 mm) and its known distribution in Cyprus below.

**Degree of establishment.** Unknown.

**Notes.** The species was first described in Fadl et al. (2007) from specimens collected near the river Nile and was considered to be endemic to Egypt. A single specimen was collected in Paphos from a plant nursery, under plastic sheets placed on the soil. It was probably introduced unintentionally in potted plants from Egypt but this is not confirmed. The high soil moisture and temperature in the glasshouse may have provided suitable microhabitats for the species.

### *Pheidole indica* Mayr, 1879

Fig. 6

**Literature records.** Salata et al. (2019).

**Material examined.** Suppl. material 1.

**Origin.** Indomalayan biogeographic realm.

**Habitat details.** Inhabiting urban habitats including parks, parking lots, pedestrian paths, plant nurseries, ports and parks as well as semi-natural habitats associated with humans such as sea and lake shores.

**Degree of establishment.** Established.

**Notes.** Synanthropic species collected from urban and semi-natural habitats in which there is human activity. In the Balearics, it [as *Ph. teneriffana*] has been observed to attack native *Tetramorium* cf. *caespitum* (Gómez and Espadaler 2006). Nevertheless, according to Sarnat et al. (2015), “*Pheidole indica* is not considered to be a major pest to either agriculture or native ecosystems”, although “further studies are required to test its ecological and agricultural impact outside its native range”.

### *Wasmannia auropunctata* (Roger, 1863)

Fig. 7

**Literature records.** Demetriou et al. (2022).

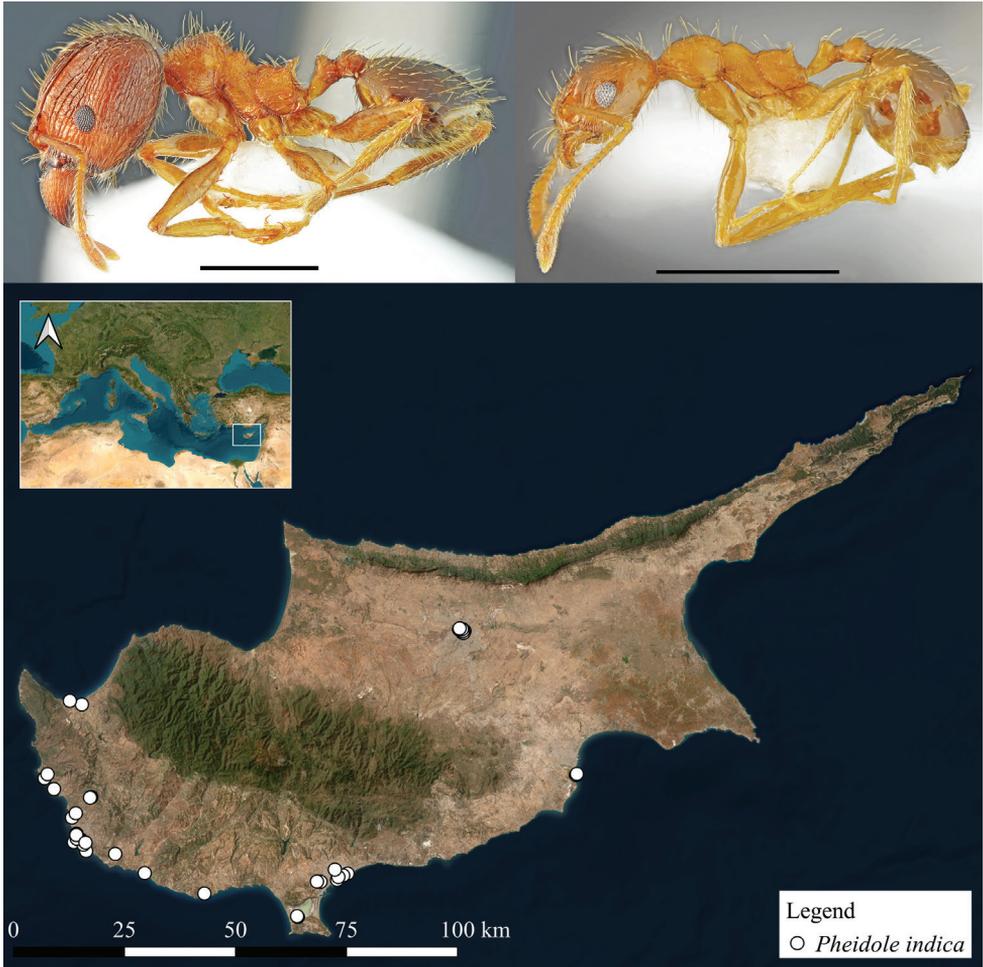
**Material examined.** Suppl. material 1.

**Origin.** Central and South America (Panamian and Neotropical biogeographic realms).

**Habitat details.** Exclusively associated with human presence. Specimens have been collected from plant nurseries, greenhouses, urban parks, parking lots and pedestrian paths near hotels and tourist sites.

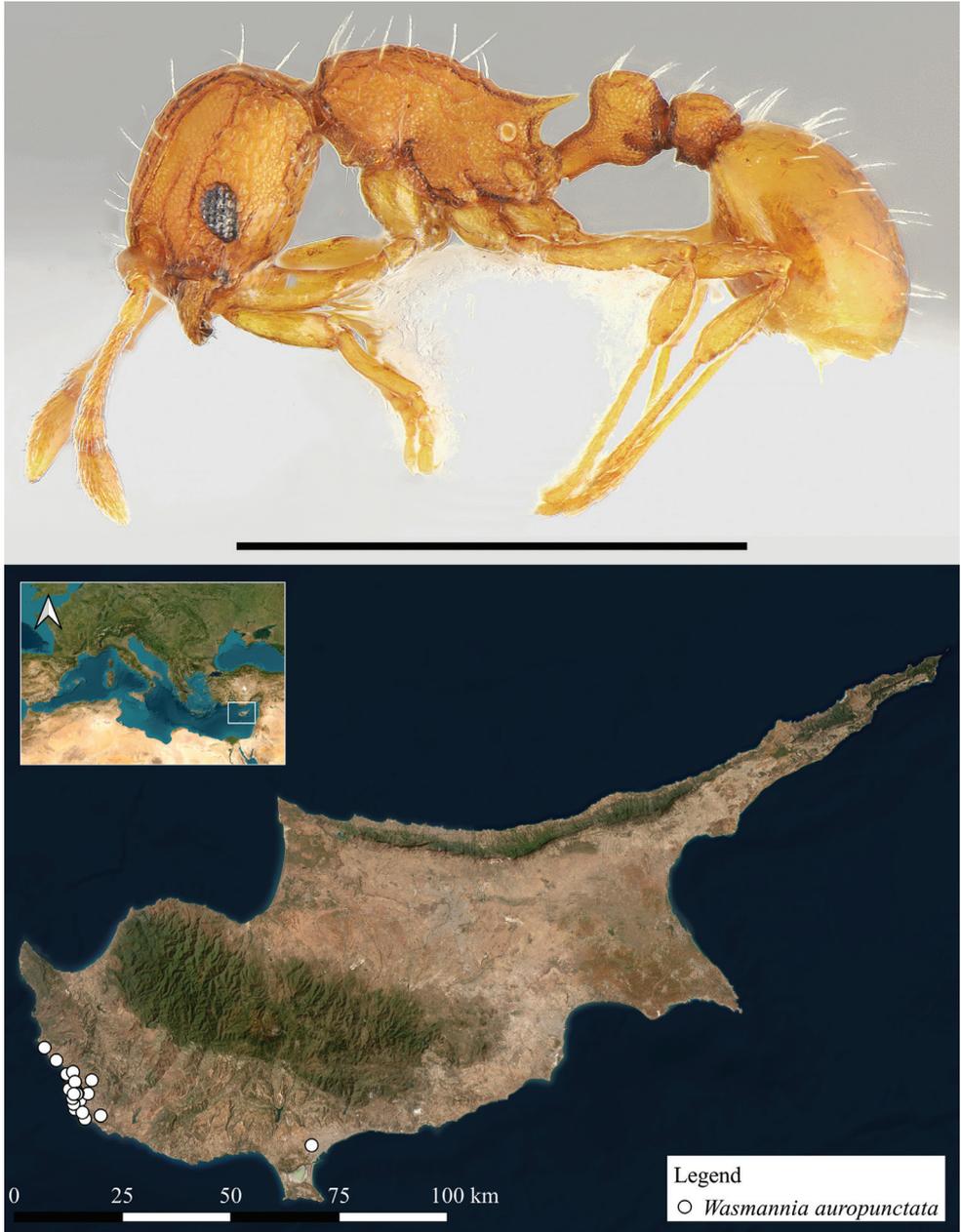
**Degree of establishment.** Established.

**Notes.** In invaded territories, *W. auropunctata* has been found to negatively affect native biodiversity as well as human and animal health (Lubin 1984; Hayashi 1999; Wetterer et al. 1999; Roque-Albelo and Causton 1999; Nishida and Evenhuis 2000; Roque-Albelo et al. 2000; Wetterer and Porter 2003; Mbenoun-Masse et al. 2017; Kidon et al. 2022). Only recently detected in Cyprus (Demetriou et al. 2022),



**Figure 6.** Habitus of *Pheidole indica* Mayr, 1879 major worker (left, from Limassol, Polemidia) and minor worker (right, Paphos, Agios Neofytos Monastery) in lateral view (scale bar: 1 mm) and its known distribution in Cyprus below.

*W. auropunctata* seems to be widely distributed in the Western part of the island (Paphos district). The abundance of the species seems to be variable, with only a few specimens collected from some sites and very high numbers from others. For example, in tourist sites, such as a hotel garden in Coral Bay and the Kato Paphos harbour, large numbers of the species were recovered while beating ornamental plants (*Hibiscus rosa-sinensis* and *Ficus microcarpa*), while sidewalks of a park in Mesogi, a hotel garden in Coral Bay and a hotel parking lot had very large colonies. More research is needed to comprehensively map the distribution of *W. auropunctata* in Cyprus and to assess interactions with native ants.



**Figure 7.** Habitus of *Wasmannia auropunctata* (Roger, 1863) (from Paphos, Kissonerga) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

**Crematogastrini*****Cardiocondyla mauritanica* Forel, 1890**

Fig. 8

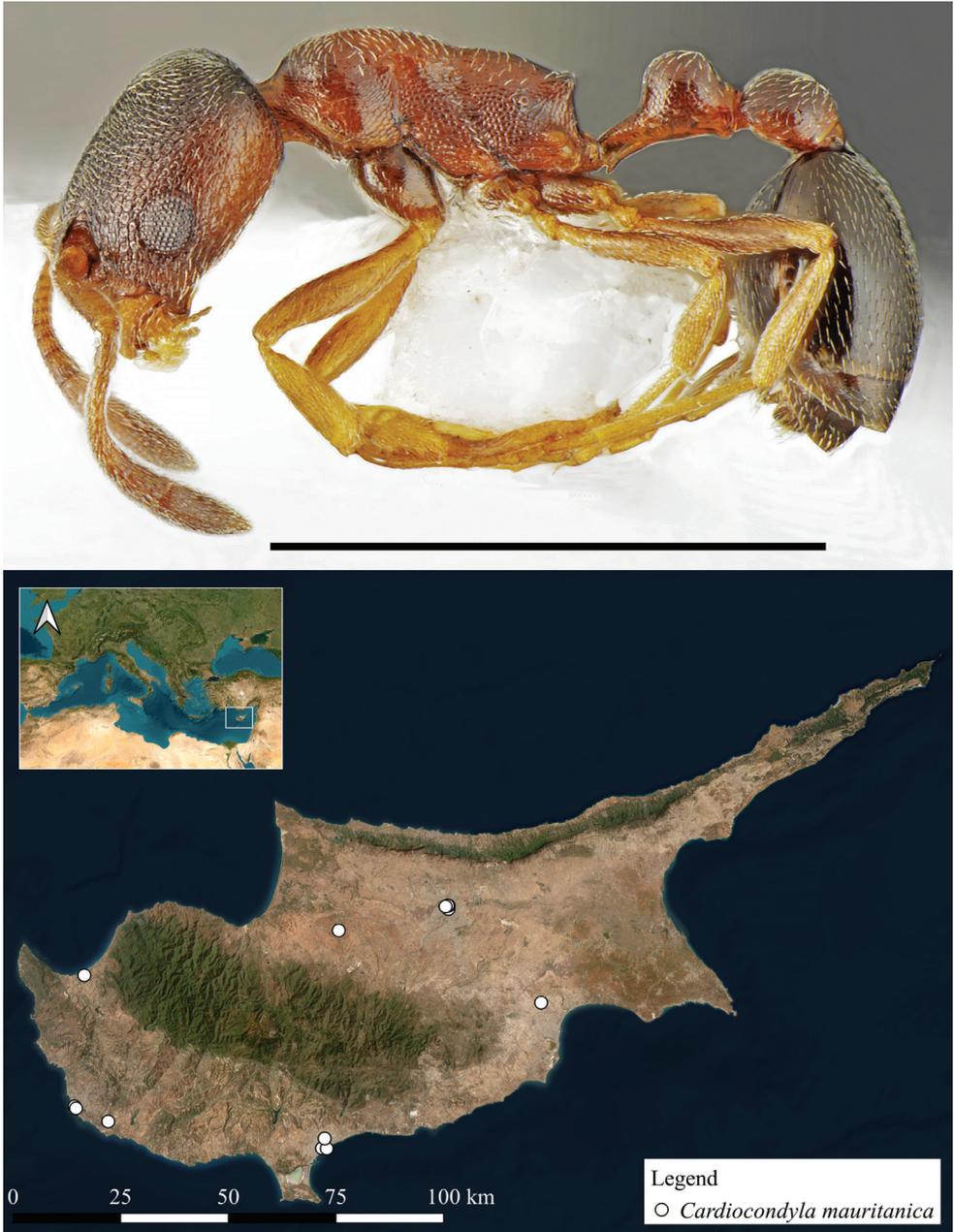
**Literature records.** Emery (1909).**Material examined.** Suppl. material 1.**Origin.** Saharo-Arabian biogeographic realm.**Habitat details.** Found in urban (park, parking lot), agricultural (plant nursery) and natural habitats (dry river bank, reservoir).**Degree of establishment.** Established.**Notes.** *Cardiocondyla mauritanica* is known to prefer xerothermous, urban and semi-arid environments (Seifert 2003; Wetterer 2014b). According to its ecological preferences, in Cyprus it has been collected from the lowlands, in disturbed areas with evident human interference.***Cardiocondyla obscurior* Wheeler, W.M., 1929**

Fig. 9

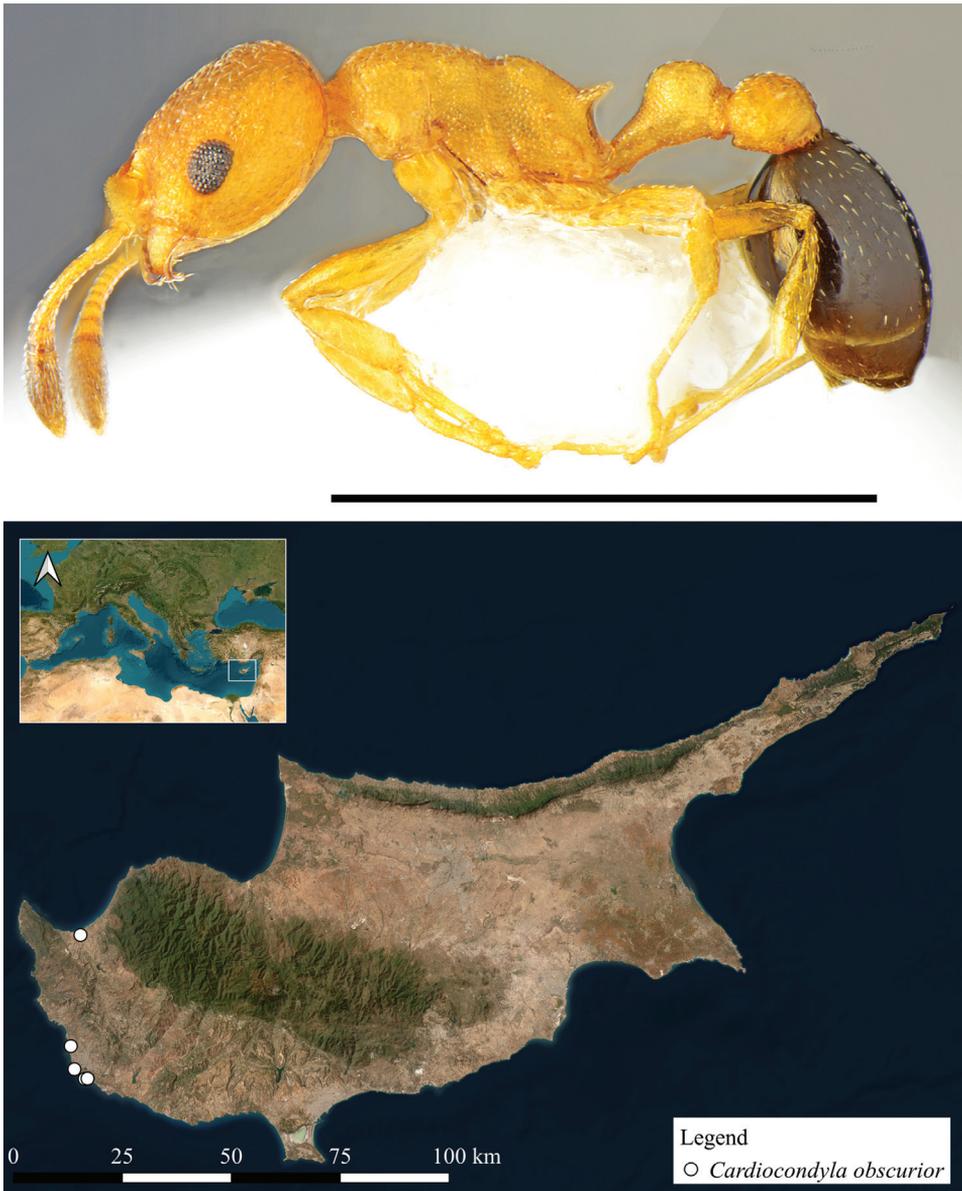
**Literature records.** N/A.**Material examined.** Suppl. material 1.**Origin.** Indomalayan biogeographic realm.**Habitat details.** Collected from urban habitats in Paphos foraging on trees, shrubs as well as on the ground in gardens and parking lots.**Degree of establishment.** Established.**Notes.** Native to Indomalaya, *C. obscurior* has been recorded from Europe and the Mediterranean as an indoor introduced species in France, Germany and the Netherlands (Seifert 2003; Blatrix et al. 2018; Boer et al. 2018) as well as an established alien species in Egypt, Israel, Syria and Spain (Donisthorpe 1930; Mohamed et al. 2001; Seifert 2003; Sánchez-García and Espadaler 2015; Janicki et al. 2016; Trigos Peral and Reyes-López 2016; Espadaler and Ortiz de Zugasti 2019). In contrast to recent findings of a single specimen in Greece (Demetriou et al. 2023b), *C. obscurior* seems to be established on the island of Cyprus with specimens being collected both on trees, according to its arboreal habits (Espadaler and Ortiz de Zugasti 2019) and from the soil.***Tetramorium bicarinatum* (Nylander, 1846)**

Fig. 10

**Literature records.** N/A.**Material examined.** Suppl. material 1.**Origin.** Indomalayan biogeographic realm.**Habitat details.** Collected from two plant nurseries and a zoo in Paphos.**Degree of establishment.** Indoor introduced.

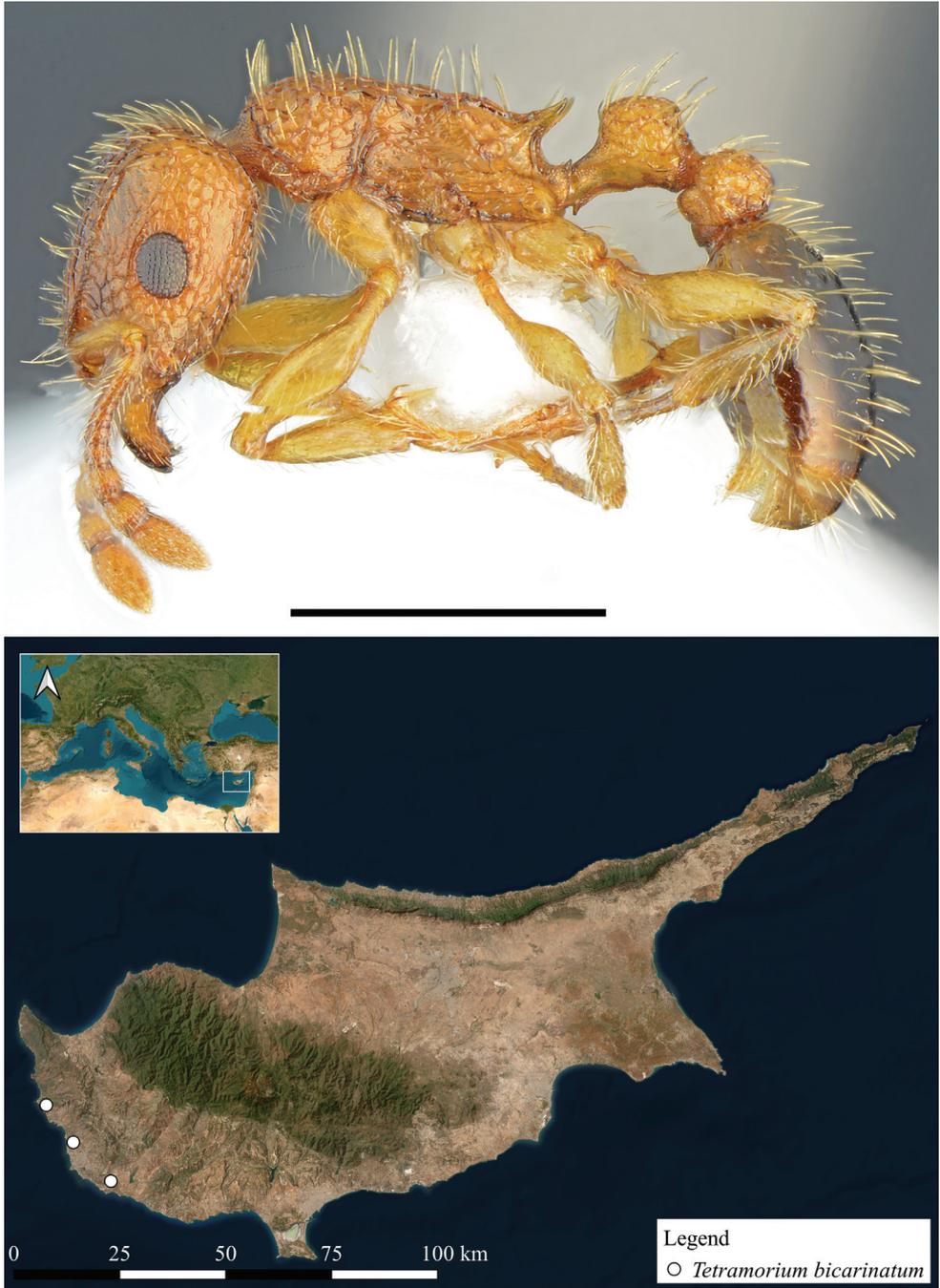


**Figure 8.** Habitus of *Cardiocondyla mauritanica* Forel, 1890 (from Limassol, Molos) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.



**Figure 9.** Habitus of *Cardiocondyla obscurior* Wheeler, W.M., 1929 (from Paphos, Kato Paphos) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

**Notes.** The distribution of this species on the island of Cyprus should be monitored. It is predicted to have socio-economic impacts on households or horticultural plants, through mutualistic relationships with aphids and scale-insects including *Aphis gossypii* Glover, 1877 (Saddiqui et al. 2019), an alien aphid species collected from a variety of arable and ornamental plants (Morris 1937; Georghiou 1977).



**Figure 10.** Habitus of *Tetramorium bicarinatum* (Nylander, 1846) (from Paphos, Paphos Zoo) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

***Tetramorium caldarium* (Roger, 1857)**

Fig. 11

**Literature records.** N/A.**Material examined.** Suppl. material 1.**Origin.** Afrotropical biogeographic realm.**Habitat details.** Found only from a zoo.**Degree of establishment.** Unknown.

**Notes.** A species that has been detected only from a few countries in the Mediterranean region, including France (Radchenko 2004), mainland Spain and the Balearic Islands (Reyes and Espadaler 2005; Gómez and Espadaler 2006) as well as Egypt (Donisthorpe 1942) and Morocco (Cagniant and Espadaler 1993; Cagniant 1997; Taheri and Reyes-López 2018) in Northern Africa. According to Bolton (1980), the species is “associated with man and living in hothouses, zoos, or other constantly heated buildings”, although it has also been collected from urban habitats in gardens, terraces, and flowerpots (Cagniant 1997) being currently widespread in the cities and coasts of Morocco (Taheri and Reyes-López 2018). In Cyprus, the species has been collected only from a zoo, during ant collection on pavements and outdoors areas. Despite its worldwide distribution, no adverse ecological impacts have been recorded for the species (Wetterer and Hirta-Garcia 2015).

***Tetramorium immigrans* Santschi, 1927**

Fig. 12

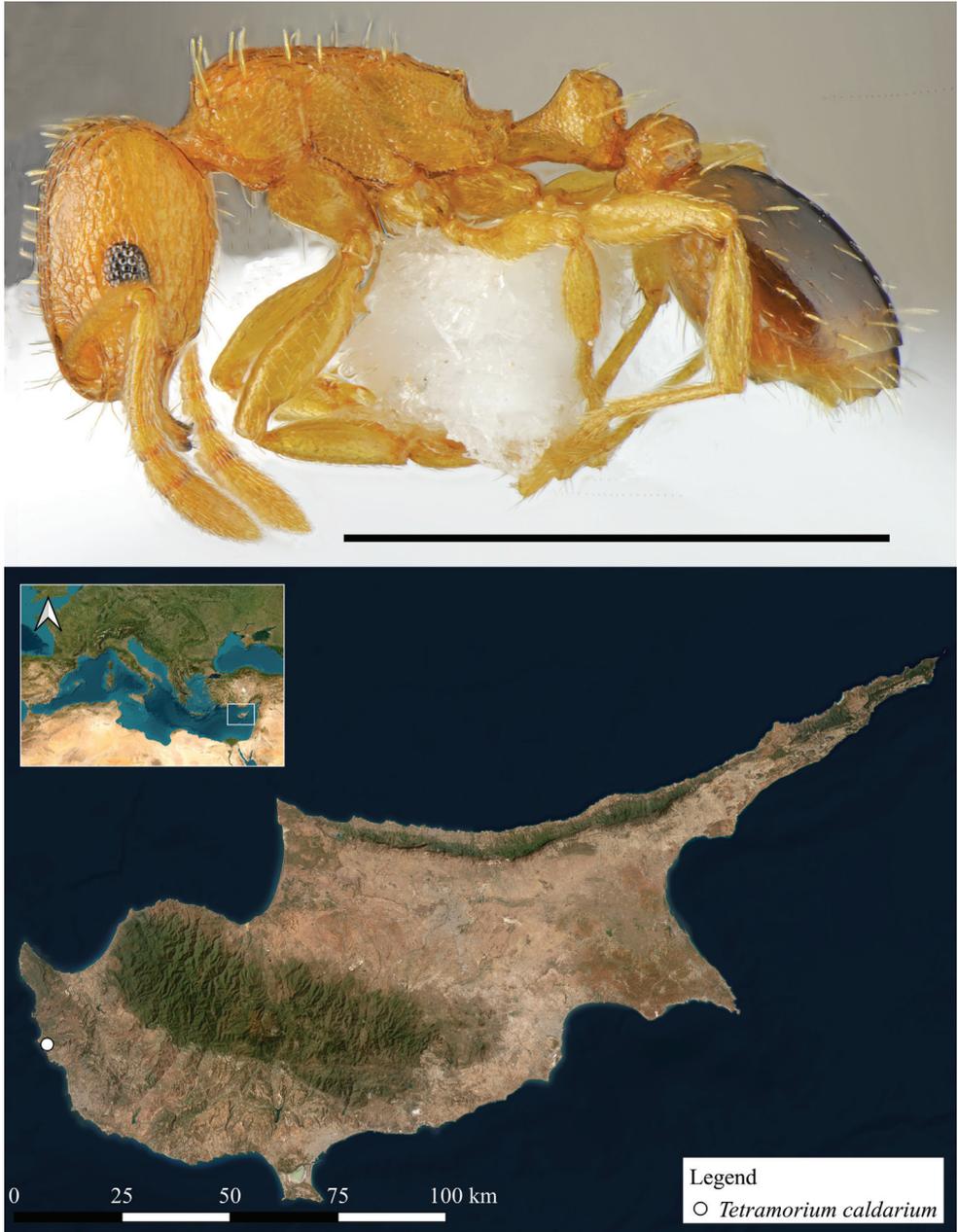
**Literature records.** N/A.**Material examined.** Suppl. material 1.**Origin.** Central Asia and Europe (Palearctic biogeographic realm).**Habitat details.** Found from only two collecting sites in Paphos district, a zoo and Kato Paphos area.**Degree of establishment.** Established.

**Notes.** A member of the recently taxonomically revised *Tetramorium caespitum* complex, currently widespread in Europe and the Mediterranean that has been found in anthropogenic and natural habitats (Wagner et al. 2017; Demetriou et al. 2023b). The species has been found to hybridise and compete with native Mediterranean species (Wagner et al. 2017; Cordonnier et al. 2019; Schifani et al. 2022). Although the native and invaded range of *T. immigrans* needs further examination, its presence in only two urban areas and absence from sampled natural habitats leads us to assume that the species is alien to the island.

***Tetramorium lanuginosum* Mayr, 1870**

Fig. 13

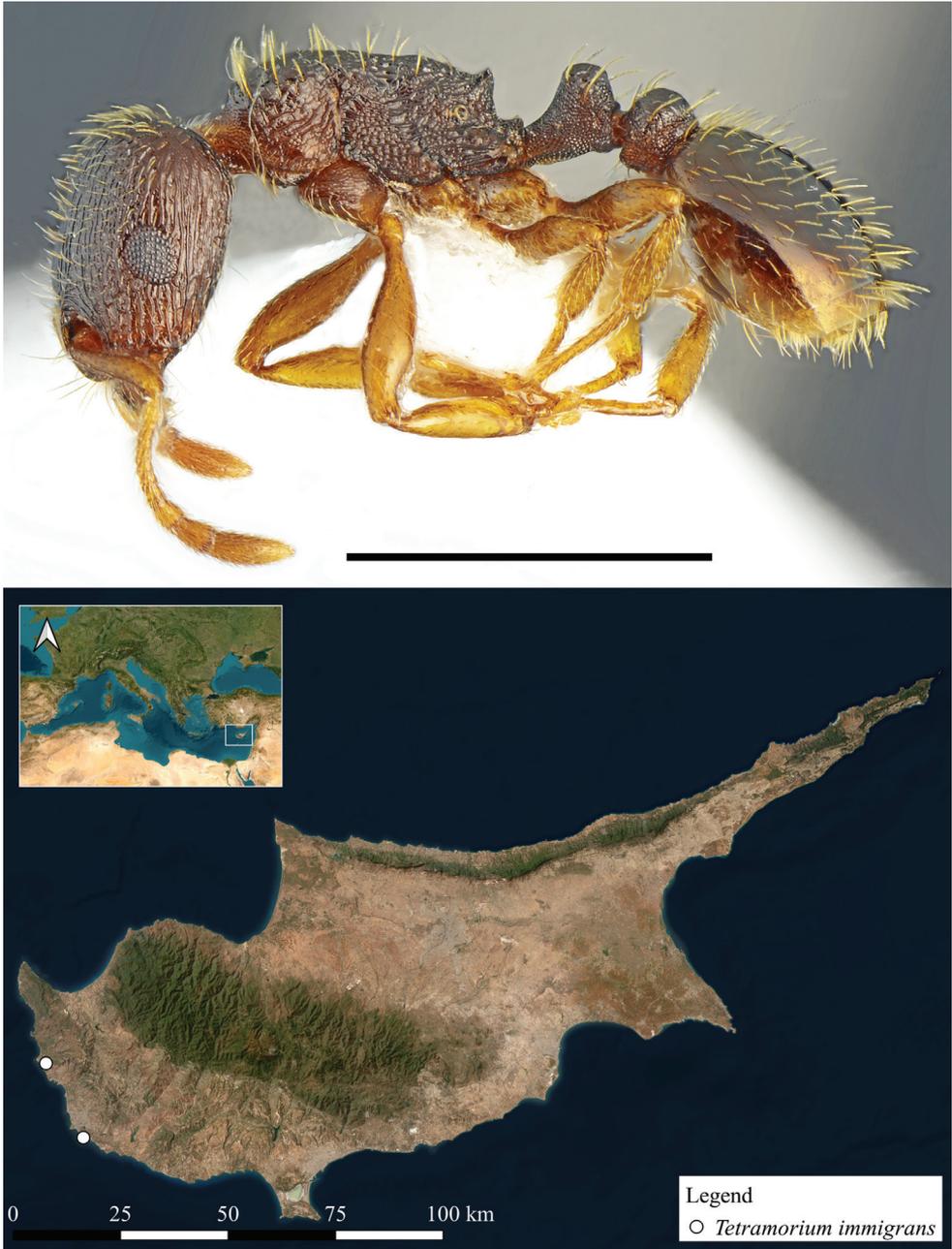
**Literature records.** N/A.**Material examined.** Suppl. material 1.



**Figure 11.** Habitus of *Tetramorium caldarium* (Roger, 1857) (from Paphos, Paphos Zoo) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

**Origin.** Subtropical East Asia (Indomalayan biogeographic realm).

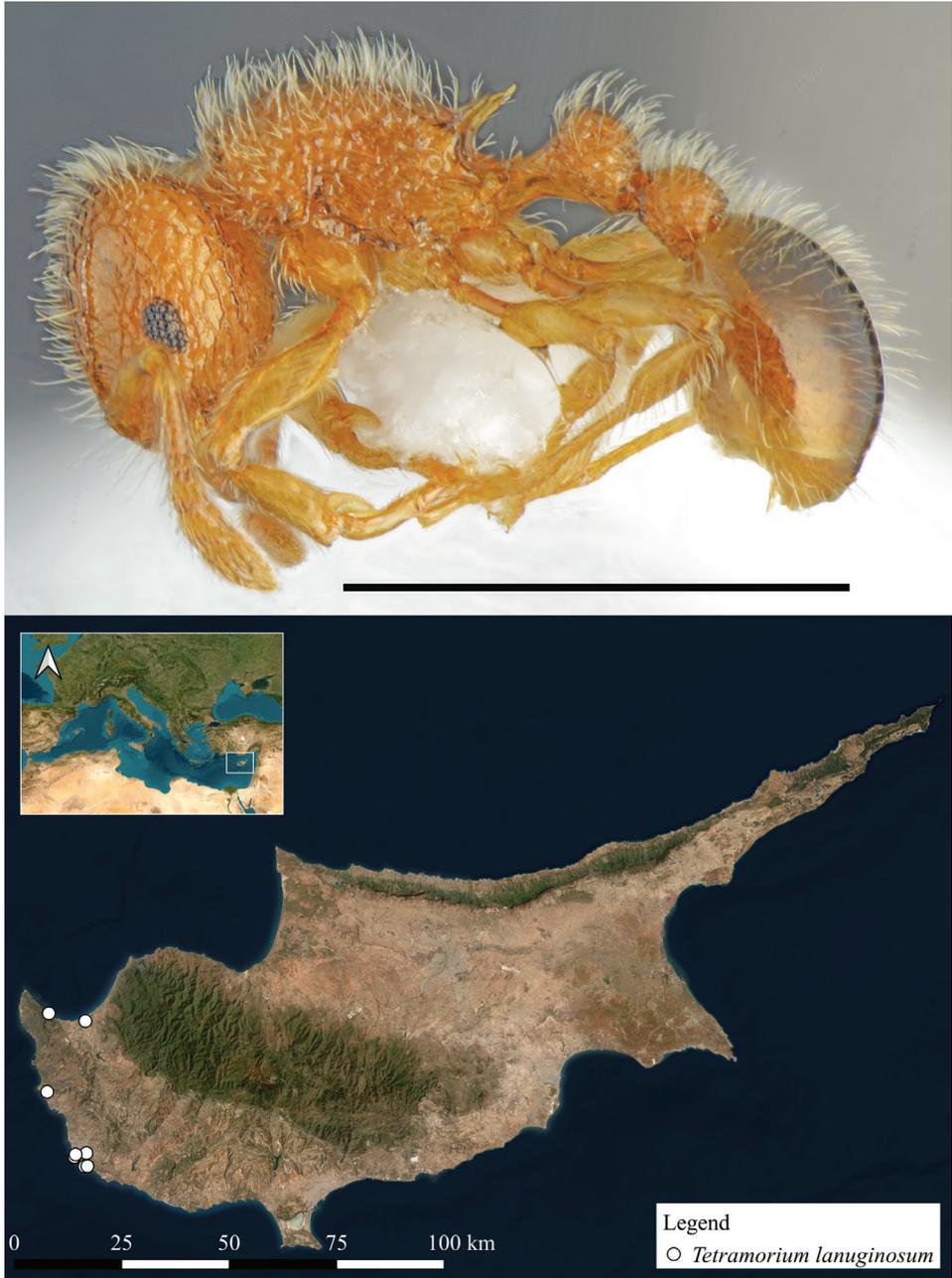
**Habitat details.** Found in urban habitats including parking lots, urban green spaces with ornamental vegetation and a zoo.



**Figure 12.** Habitus of *Tetramorium immigrans* Santschi, 1927 (from Paphos, Paphos Zoo) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

**Degree of establishment.** Established.

**Notes.** The species has been collected from neighbouring Israel and Lebanon since the last century and more recently from Egypt (Wetterer 2010a). In Spain, *T. lanuginosum* was



**Figure 13.** Habitus of *Tetramorium lanuginosum* Mayr, 1870 (from Paphos, Paphos Zoo) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

first recorded by Reyes and Espadaler (2005) and subsequently collected from a date palm grove where it presumably did not establish (Casiraghi et al. 2020). *Tetramorium lanuginosum* has been also recorded from urban habitats in Malta and Sicily (Schembri and Colling-

wood 1981; Schifani and Alicata 2018). In Cyprus it can be found in anthropogenic habitats, with two sites within the boundaries of the protected Akamas Peninsula (CY4000023 and CY4000010). Although the species has no recorded adverse environmental impacts, more research is necessary to establish its further spread into protected areas.

## Solenopsidini

### *Monomorium bicolor* Emery, 1877

Fig. 14

**Literature records.** Salata and Borowiec (2015b); Salata et al. (2019).

**Material examined.** Suppl. material 1.

**Origin.** Africa, Arabian Peninsula, Levant and Turkey (Palearctic, Saharo-Arabian and Afrotropical biogeographic realms).

**Habitat details.** Widespread, found in all kinds of habitats.

**Degree of establishment.** Established.

**Notes.** The most common and widespread alien ant in Cyprus, reaching the highest altitudinal range and collected from 15 protected areas. Despite its large AOO and EOO, it has been only recently detected in Cyprus (Salata and Borowiec 2015b) and does not seem to have any adverse environmental impacts. Nevertheless, the alien or native status of this species in the Middle East and in Cyprus is rather problematic. *Monomorium bicolor* is believed to be native to the Afrotropics (Bolton 1987) although on AntMaps it is shown as native to the whole of Africa and Middle East except Greece and Cyprus. Despite its recent report from Cyprus, its distribution on the island may suggest either its unintentional introduction since ancient times (L. Borowiec pers. comm.) or indeed its native status. Molecular analyses on populations from Cyprus, the Middle East, Northern and Sub-Saharan Africa could help to unveil the true status of the species in the aforementioned regions.

### *Solenopsis* sp\_CYP139

Fig. 15

**Literature records.** N/A.

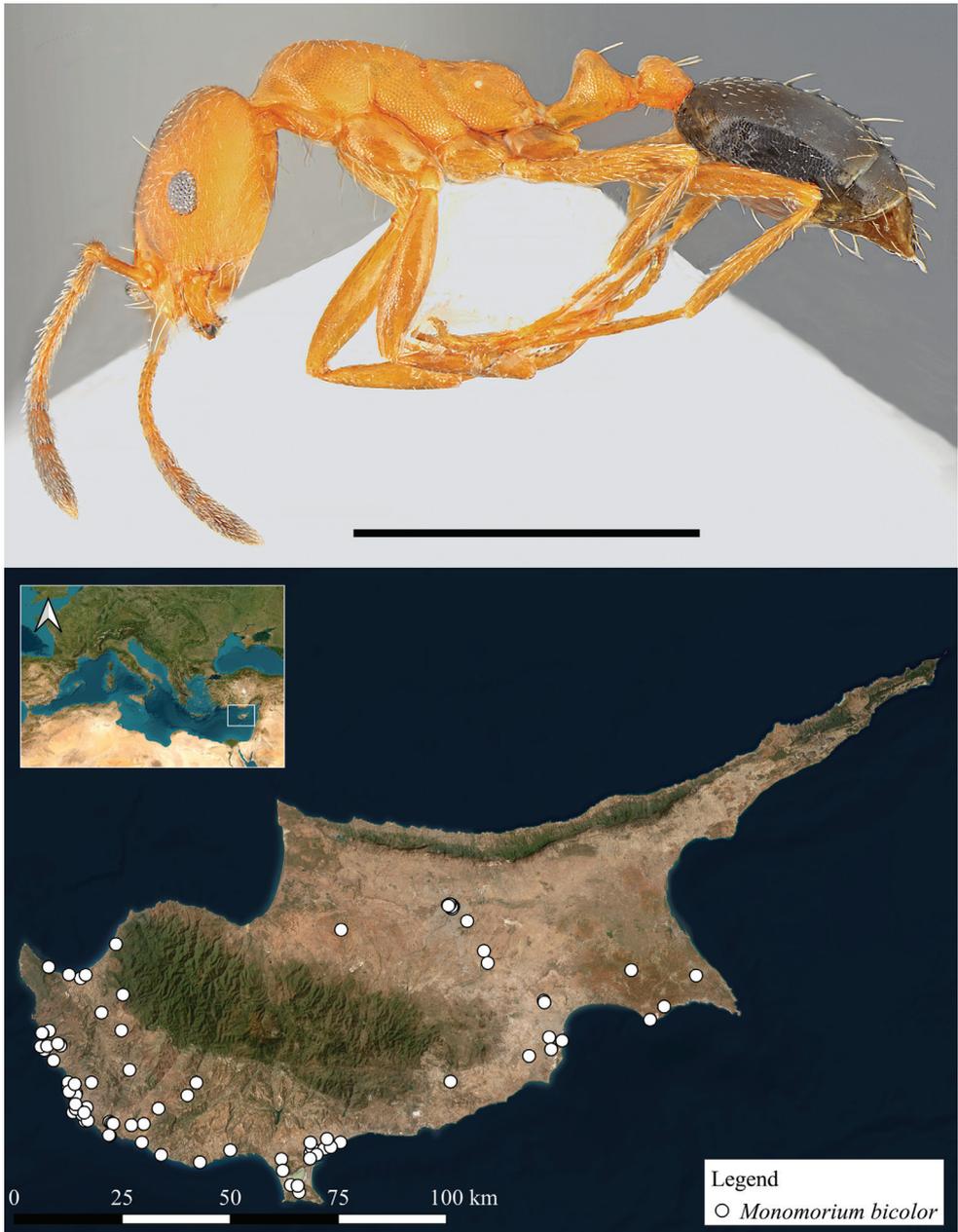
**Material examined.** Suppl. material 1.

**Origin.** Unknown.

**Habitat details.** Collected from plant nurseries (indoors in crops under foil) and outdoor urban areas of Limassol and Paphos.

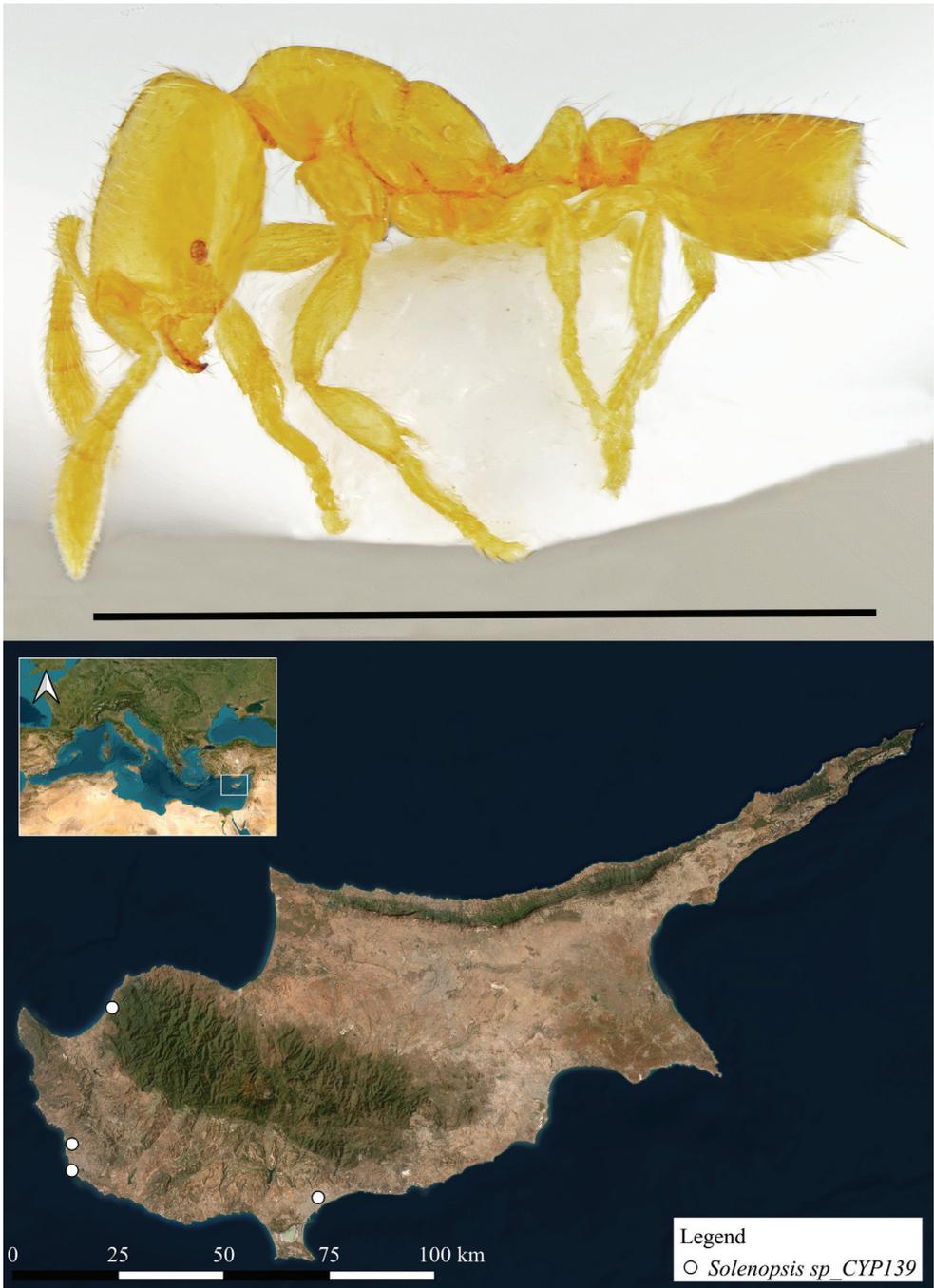
**Degree of establishment.** Established.

**Notes.** This unidentified species is well distinguished from all native species from Greece and Turkey; it is very small in size and has extremely small eyes (in the form of a black dot). We have decided to classify this morpho-species as alien due to its occurrence only within urban habitats and plant nurseries, which lead us to the hypothesis of an unintentional introduction via infested plant material. The species presumably



**Figure 14.** Habitus of *Monomorium bicolor* Emery, 1877 (from Paphos, Kato Paphos) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

belongs to an unidentified species of tropical/subtropical origin, which could be further investigated with the help of molecular tools. The description and morphometric data of this morphospecies can be found Suppl. material 4 for further examination and future comparisons.



**Figure 15.** Habitus of *Solenopsis* sp\_CYP139 (from Paphos, Lemba) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

***Trichomyrmex destructor* (Jerdon, 1851)**

Fig. 16

**Literature records.** Georghiou (1977); Wetterer (2009b).**Material examined.** Suppl. material 1.**Origin.** Indomalayan biogeographic realm.

**Figure 16.** Habitus of *Trichomyrmex destructor* (Jerdon, 1851) (from Limassol, Savvas Savva park) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

**Habitat details.** Urban habitats including houses, parking lots and paved roads.

**Degree of establishment.** Established.

**Notes.** Wetterer (2009b) cite “CAB 2000 in DAFF 2001” as a literature source for the presence of *T. destructor* in Cyprus. When checking CABI (2022) *T. destructor* was reported as present in Cyprus citing “CABI Data Mining (Undated)”. Despite comments of Salata et al. (2019) excluding the species from their list, Demetriou et al. (2023a) decided to include it based on records of Georghiou (1977), which had previously been overlooked. In particular, Georghiou (1977) noted this species as “*Monomorium destructor* Jerd.”, “from olives infested by *Dacus oleae*” in 1956 as well as in 1925 under the synonymic name “*M. gracillimum* Sm.” “feeding of eggs of *Thaumatopeoa wilkinsoni* (Lep., Notodontidae)”. Upon collection of further material we confirm the presence of *T. destructor* on the island. The species was collected from five urban sites in Paphos and Limassol districts. *Trichomyrmex destructor* is known to be associated with electronic devices causing electrical damages as well as destroying stored household products and inflicting painful bites on humans (Wetterer 2009b). Negative socio-economic impacts in households or possible health risk could be monitored through citizen science initiatives assessing the socio-economic impacts of ants in households.

***Trichomyrmex mayri* (Forel, 1902)**

Fig. 17

**Literature records.** N/A.

**Material examined.** Suppl. material 1.

**Origin.** Indomalayan biogeographic realm [speculated by Bolton (1987)].

**Habitat details.** Both in urban (dirt roads, road sides and uncultivated green spaces) and natural habitats (dry meadow, pine forest, reservoir).

**Degree of establishment.** Established.

**Notes.** A species common in the Arabian Peninsula (Sharaf et al. 2013), which is believed to originate from Indomalaya (Bolton 1987). It has been recorded from neighbouring Egypt, Israel and Syria where it is considered alien (Bolton 1987). In Cyprus, it occupies a large EOO and altitudinal range, while it has been also recorded from three protected areas (CY2000004, CY2000013 and RAMSAR1375). Nevertheless, its potential environmental or socio-economic impacts are unknown both throughout its native and introduced range.

**Ponerinae**

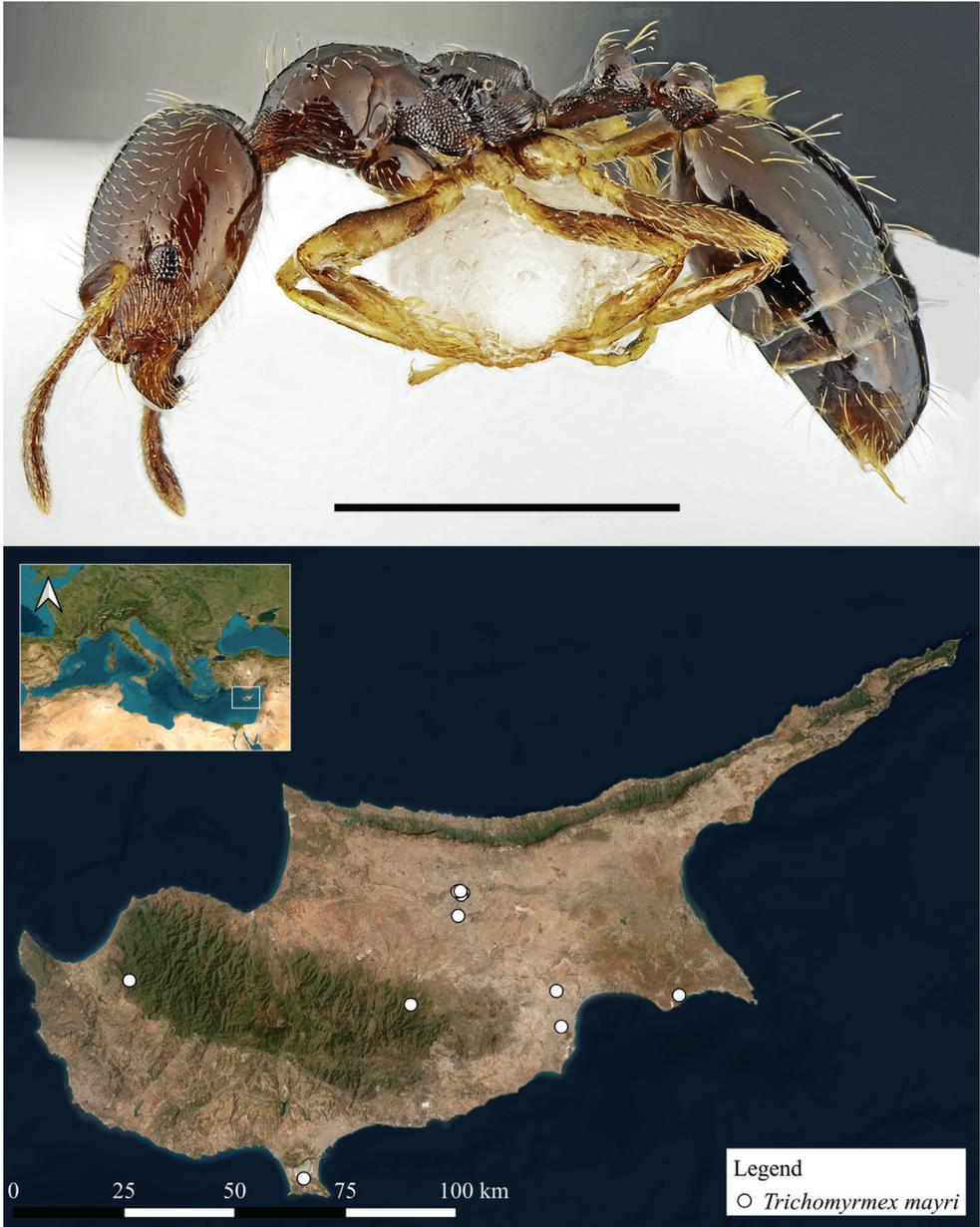
**Ponerini**

***Hypoponera punctatissima* (Roger, 1859)**

Fig. 18

**Literature records.** N/A.

**Material examined.** Suppl. material 1.

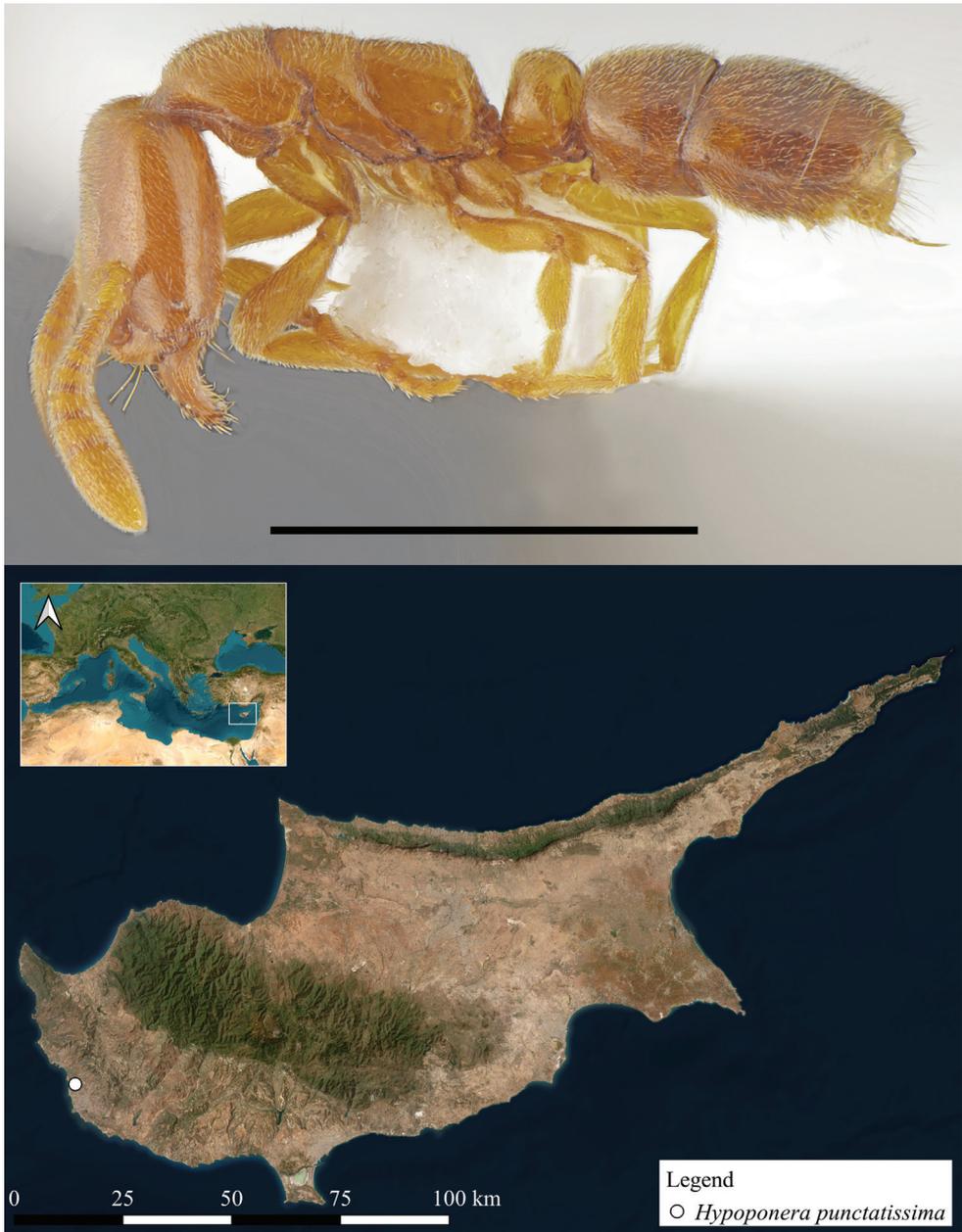


**Figure 17.** Habitus of *Trichomyrmex mayri* (Forel, 1902) (from Paphos, Lysos vic.) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

**Origin.** Probably Egypt and Sub-Saharan Africa (Saharo-Arabian and Afrotropical biogeographic realms).

**Habitat details.** Only one collection site corresponding to a plant nursery (indoors in crops under foil) in Paphos.

**Degree of establishment.** Unknown.



**Figure 18.** Habitus of *Hypoponera punctatissima* (Roger, 1859) (from Paphos, Lemba) in lateral view above (scale bar: 1 mm) and its known distribution in Cyprus below.

**Notes.** An alien species collected across Europe and the Mediterranean from both indoors and outdoors localities, including natural habitats (Atanassov and Dlusskij 1992; Czechowski et al. 2012; Borowiec and Salata 2022). Although its native range remains quite uncertain, its presence on the island is supported by only one specimen collected in a plant nursery. This may indicate its recent introduction to the island

through the horticultural or ornamental pathway, with further spread leading to its establishment in areas with sufficient humidity as observed in other European countries. Although *Hypoponera ergatandria* Forel, 1893 has been also noted as an alien species in Europe, the status of this species is uncertain, representing either a valid species or a junior synonym of *H. punctatissima*. As such, *H. ergatandria* is not included in the provided identification key and considered here as a junior synonym of *H. punctatissima* (Bolton and Fisher 2011; Seifert 2013; Lubertazzi 2019).

### Species excluded from previous checklists

#### *Cardiocondyla nuda* (Mayr, 1866)

**Notes.** Although previously thought to be a widespread alien species, a revision of the group has concluded that *C. nuda* is geographically restricted in Oceania (Seifert et al. 2017). According to Seifert (2003), investigation of authentic material from the Palearctic turned out to be *C. mauritanica*. Although the species is mentioned for Cyprus by Bernard (1956), these records also probably correspond to *C. mauritanica*.

#### *Monomorium pharaonis* (Linnaeus, 1758)

**Notes.** The only records of the species can be traced back to Wetterer (2010b) citing Radchenko (2004), a record corresponding to data in the Fauna Europaea database. Nevertheless, no specimens or scientific literature have been provided to support these records. The record of *M. pharaonis* in Fauna Europaea is probably erroneous as already shown for other ant species in the database, noted for example by Schifani and Alicata (2018) regarding Sicily or Demetriou et al. (2023b) for Greece. Although the presence of *M. pharaonis* on the island is highly possible, given its extended distribution throughout the world (Wetterer 2010b), we tentatively suggest the exclusion of the species from lists of alien species of Cyprus until further investigations provide adequate evidence. Citizen science approaches looking into the biodiversity and socio-economic impacts of ants in households could potentially uncover records of this synanthropic, indoors introduced species.

#### *Monomorium subopacum* (F. Smith, 1858)

**Notes.** As in the case of Greece (Demetriou et al. 2023b), following assumptions of Bolton (1987) and the native status of the species for neighbouring Mediterranean countries (AntWiki 2022), *M. subopacum* is considered native to Cyprus.

#### *Nylanderia vividula* (Nylander, 1846)

**Notes.** In Georghiou (1977) "*Paratrechina nitidula* Nyl." is mentioned as collected in 1935 and identified by H.S. Donisthorpe. Such a nomenclatural combination does not exist and probably corresponds to *Paratrechina vividula* (Nylander, 1846), currently an

obsolete combination of *N. vividula*. *Nylanderia vividula* has been reported as an alien species from neighbouring Egypt, Greece, Israel, Lebanon, and Turkey (Emery 1881, 1898; Donisthorpe 1950; Borowiec 2014). However, records from Greece are believed to correspond to *N. jaegerskioeldi* (Demetriou et al. 2023b) and no additional records have been uncovered from Turkey since Donisthorpe (1950). During our sampling in Cyprus we extensively collected only samples of *N. jaegerskioeldi*. *Nylanderia vividula*, a species native to the Nearctic biogeographic realm, might have been introduced to the island but failed to establish. Alternatively, records may correspond to *N. jaegerskioeldi*, a common species missing from Georghiou's (1977) list but already known from Cyprus since 1910 (Emery 1910). Further sampling and tracing material in historical collections may shed light on the presence and invasion history of *N. vividula* on Cyprus. For the time being, records from Cyprus remain doubtful and in need of verification.

### ***Solenopsis geminata* (Fabricius, 1804)**

**Notes.** The only record mentioning the presence of *S. geminata* in Cyprus can be traced to Collingwood et al. (1997), where the island is simply listed in the known distribution of the species. After 25 years of this mention no material has been uncovered. As in the case of Greece (Demetriou et al. 2023b), we expect that the presence and impacts of this invasive alien species would not go unnoticed. In the possible event of its past introduction to the island, any individuals or populations did not manage to establish. Furthermore, as horizon scanning exercises undertaken on the island (Peyton et al. 2019) did not assess *S. geminata*, due to the aforementioned dubious records, the possibility of the species reaching Cyprus, establishing viable populations and impacting native biodiversity, could be taken into consideration in the future.

### ***Hypoponera eduardi* (Forel, 1894)**

**Notes.** Although included in the list of invasive ants of Greece and Cyprus (Salata et al. 2019), upon re-examination the species is currently treated by the authors as native to Cyprus due to both its narrow ecological requirements (i.e. soil moisture, habitat types found) as well as its native status in neighbouring countries (Demetriou et al. 2023b).

## **Identification key to distinguish alien from native worker ants inhabiting Cyprus, with the support of male and queen characters**

### **Key to subfamilies**

[After Borowiec and Salata (2022), modified]

- 1 Pedicel with two distinct segments (petiole and postpetiole) (Suppl. material 3: figs S56, S58, S60)..... **Myrmicinae**
- Pedicel with one segment (petiole) (Suppl. material 3: figs S3, S16, S20)..... **2**

- 2 Sting projected, first gastral segment separated from the second one by a distinct constriction (Suppl. material 3: figs S142, S144, S146, S148).....**3**
- Sting not projected, first and second gastral segments not separated by a constriction (Suppl. material 3: figs S16, S18, S20, S22).....**5**
- 3 Tergite of the second gastral segment much longer than its sternite, strongly arched, abdominal segments pointed downward .... **Proceratiinae** (only one native species)
- Tergite of the second gastral segment as long as its sternite, never arched, abdominal segments not pointed downward.....**4**
- 4 Petiole broadly attached to the first gastral segment, separated from it only by shallow constriction ..... **Amblyoponinae** (only one native species)
- Petiole narrowly attached to the first gastral segment, separated from gaster by sharp and deep constriction (Suppl. material 3: figs S142, S144, S146, S148) ....  
.....**Ponerinae**
- 5 Apex of gaster with circular nozzle-like acidopore, fringed with setae (Suppl. material 3: figs S18, S20, S22) .....**Formicinae**
- Apex of gaster without acidopore and coronula only with transverse slit (Suppl. material 3: figs S1, S3, S5) ..... **Dolichoderinae**

### Subfamily Dolichoderinae

[After Salata and Borowiec (2022), and Fisher and Bolton (2016), modified]

- 1 Petiole very reduced or vestigial, in profile very small and low, often invisible from above, covered under the first segment of the gaster (Suppl. material 3: figs S1, S3, S5, S7) .....**2**
- Petiole a well-developed scale, distinct in profile (Suppl. material 3: figs S14, S16)....**5**
- 2 Anterior margin of clypeus straight (Suppl. material 3: fig. S2); in dorsal view 5 gastral tergites visible, the fifth gastral tergite small but continues the line of the gaster and is not reflexed below the fourth; gastral tergites with numerous erect and thick setae (Suppl. material 3: fig. S1) ..... **Technomyrmex** (not reported from Cyprus but possible one alien outdoor or five alien indoor species known from Europe)
- Anterior margin of clypeus in the middle shallowly or deeply emarginate (Suppl. material 3: figs S4, S6, S8, S13); in dorsal view only 4 gastral tergites visible, the fifth gastral tergite reflexed below the fourth; gastral tergites without erect and thick setae (Suppl. material 3: figs S3, S5, S7, S12) .....**3**
- 3 Large species, mesosoma length in major workers above 1 mm; body uniformly brown to black (Suppl. material 3: figs S3, S5, S7) anterior margin of clypeus with distinct median emargination (Suppl. material 3: figs S4, S6, S8).....**4**
- Very small, mesosoma length always below 0.6 mm. Body bicoloured, head brown to black, mesosoma and anterior half or gaster with pale, whitish to whitish-brown areas (Suppl. material 3: fig. S12). Anterior margin of clypeus with very shallow median emargination (Suppl. material 3: fig. S13) .....  
.....**Tapinoma melanocephalum** (alien species not reported from Cyprus)

- 4 Parameres in male genitalia with broadly rounded apex (Suppl. material 3: fig. S10)..... ***Tapinoma magnum*** (native to Western Europe but alien in Greece, not reported from Cyprus)
- Parameres in male genitalia with narrow or distinctly angulate apex (Suppl. material 3: figs S9, S11)..... ***Tapinoma*** (two native species)
- 5 Propodeum in profile not or only slightly surpassed by promesonotum (Suppl. material 3: fig. S14) mandibular denticles not strongly heterodont (Suppl. material 3: fig. S15) .....***Bothriomyrmex*** (one native species)
- Propodeum in profile distinctly surpassed by promesonotum (Suppl. material 3: fig. S16) mandibular denticles strongly heterodont, with 5–6 larger dents dispersed over the masticatory border and 11–15 clearly smaller denticles present in interspaces (Suppl. material 3: fig. S17).....  
.....***Linepithema humile*** (alien species not reported from Cyprus)

**Subfamily Formicinae**

[After Borowiec and Salata (2022), modified]

- 1 Antennae 11-segmented..... **2**
- Antennae 12-segmented..... **5**
- Antennae 9-segmented.....  
.....***Brachymyrmex patagonicus*** (alien species not recorded from Cyprus)
- 2 Propodeum unarmed, rounded in profile, dorsal margin of petiole not emarginate (Suppl. material 3: figs S20, S22, S28)..... **3**
- Propodeum bispinose or bituberculate, dorsal margin of petiole emarginated (Suppl. material 3: figs S18) ..... ***Lepisiota*** (at least three native species)
- 3 Very small ants, legs and antennae short, hind femora not extending behind apex of gaster (Suppl. material 3: figs S22–S25) ..... **4**
- Moderately large ants with very elongate legs and antennae, hind femora distinctly extending behind the apex of gaster (Suppl. material 3: figs S20, S21) .....  
.....***Anoplolepis gracilipes*** (alien species not reported from Cyprus)
- 4 Body yellowish-brown to dark brown, if mostly yellow then first gastral tergite without dark spots in posterolateral corners (Suppl. material 3: figs S24, S25) ...  
***Plagiolepis*** (at least three native species and their two workerless social parasite species)
- Body mostly yellow, only posterolateral corners of first gastral tergite and apex of gaster with brown spots (Suppl. material 3: figs S22, S23) .....  
.....***Plagiolepis alluaudi*** (alien indoor species not reported from Cyprus)
- 5 Antennal insertions placed distinctly behind clypeal margin (Suppl. material 3: figs S27, S29, S31, S33) ..... **6**
- Antennal insertions placed close to clypeal margin (Suppl. material 3: figs S41, S43, S45, S47, S49) ..... **10**

- 6 Frontal carinae straight, slightly converging to the front in major workers, head truncate anteriorly, plug-like (Suppl. material 3: figs S26, S27) ..... **Colobopsis** (one native species)
- Frontal carinae arched, distinctly converging to the front in major workers head not modified (Suppl. material 3: figs S31, S33, S37) ..... 7
- 7 Dorsal profile of mesosoma with deep impression between mesonotum and propodeum; propodeum always abruptly falling down to its caudal slope (Suppl. material 3: fig. S28)..... **Camponotus subgen. Myrmentoma**, part (two native species)
- Dorsal profile of mesosoma continuously convex or with only shallow impression between mesonotum and propodeum (Suppl. material 3: figs S32, S34, S36, S38) ..... **8**
- 8 Anterior margin of clypeus forms regular arch, without any protrusion (Suppl. material 3: figs S31, S33, S35) ..... **9**
- Anterior margin of clypeus forms a lobiform protrusion extending beyond anterior margin of gena (Suppl. material 3: figs S37, S39) ..... **Camponotus subgen. Tanaemyrmex**, part (five native species)
- 9 Body completely black; dorsum of propodeum flat or slightly convex (Suppl. material 3: figs S32–S35) ..... **Camponotus subgen. Myrmentoma, part** (two native species)
- Body colouration various, head, mesosoma and femora partly reddish, partly reddish-brown to brown; propodeum with dorsal concavity (Suppl. material 3: figs S30, S31) ..... **Camponotus cf. vitiosus** (alien species)
- 10 Propodeal spiracle round to oval; legs short to moderately long (Suppl. material 3: figs S42, S44, S46), HTL/CW < 1.50 ..... **11**
- Propodeal spiracle elongate; legs very long (Suppl. material 3: fig. S40), HTL/CW > 1.50 ..... **Cataglyphis** (two native species)
- 11 Eyes situated at or in front of the midlength of the sides of head (Suppl. material 3: figs S43, S45) ..... **12**
- Eyes situated distinctly behind the midlength of the sides of head (Suppl. material 3: figs S41, S47) ..... **13**
- 12 Mandible unsculptured, with 6–7 teeth; scape short, less than 1.5 times length of head, body stout (Suppl. material 3: fig. S43)..... **Nylanderia jaegerskioeldi** (alien species)
- Mandible with longitudinal striation and 5 teeth; scape long, more than 1.5 times length of head, body elongate (Suppl. material 3: fig. S47)..... **Paratrechina longicornis** (alien species)
- 13 Hind coxa widely separated; orifice of propodeal spiracle circular or broadly oval; mesosoma rather short and high, usually densely pubescent; propodeum approximately two times shorter than high (Suppl. material 3: figs S48, S50, S52). Size usually smaller, total length 2.5–5.0 mm ..... **14**
- Hind coxa close together; orifice of propodeal spiracle elongate to slit-like; mesosoma rather long and slender, usually sparsely pubescent (Suppl. material 3: fig. S46); propodeum approximately as long as high (Suppl. material 3: fig. S46). Size larger, total length 4.5–9.0 mm ..... **Formica** (one native species)

- 14 Pubescence on the whole body present but moderate to dense; antennal scapi lacking erect setae; pubescence of clypeus sparse, surface of the clypeus well visible (Suppl. material 3: figs S48, S49); mean number of mandibular dents < 8 ..... *Lasius neglectus* (alien species not reported from Cyprus)
- Characters combination different (Suppl. material 3: figs S50–S53) ..... *Lasius* (two native species)

**Subfamily Myrmicinae**

[After Salata et al. (2020), Borowiec and Salata (2022), Bolton (1980, 2000) and Seifert (2023), modified]

- 1 Postpetiole attached to dorsum of first gaster segment, petiole without node (Suppl. material 3: figs S54, S55) ..... *Crematogaster* (five native species)
- Postpetiole attached to anterior part of first gaster segment, petiole with node (Suppl. material 3: figs S56, S58, S60) ..... 2
- 2 Antennae 10- or 11-segmented ..... 3
- Antennae 12-segmented ..... 9
- Antennae 4 to 6-segmented ..... *Strumigenys* (no species recorded from Cyprus. If dorsal alitrunk and first gastral segment without erect hair then *S. membranifera* – alien species)
- 3 Antennae 11-segmented; two prominent propodeal spines (Suppl. material 3: figs S56, S58) ..... 4
- Antennae 10-segmented; no propodeal spines (Suppl. material 3: figs S60, S62, S64, S66, S68, S70) ..... 5
- 4 Eyes big, longitudinal, narrowing downwards, anterior margin of eye situated very close to insertion of mandible (Suppl. material 3: figs S56, S57); head without antennal scrobes. Body colour black ..... *Oxyopomyrmex* (one native species)
- Eyes moderate, elongate oval, situated in distance from insertion of mandible; head with antennal scrobes; body colour yellow to rusty (Suppl. material 3: figs S58, S59) ..... *Wasmannia auropunctata* (alien species)
- 5 Body colour pale, yellow, occipital margin of head straight or shallowly emarginate; length of mesosoma in major workers below 1 mm (Suppl. material 3: figs S62–S65) ..... 6
- Body colour orange-red to black, if yellow then head of large majors with deeply emarginate occipital margin; length of mesosoma in major workers distinctly above 1 mm (Suppl. material 3: figs S66–S71) ..... 7
- 6 Larger species, in major workers, length of mesosoma up to 0.7 mm or small species (length of mesosoma < 0.55 mm) with elongated head (head length/width ratio 1.3–1.4) (Suppl. material 3: figs S62, S63) ..... *Solenopsis* (two native species)
- Small species (ML < 0.55 mm), head stouter (head length/width ratio < than 1.3) (Suppl. material 3: figs S64, S65) ..... *Solenopsis* sp\_CYP139 (one unidentified alien indoor species)

- 7 Body yellow to reddish-brown, sometimes with mixed yellow and brown, only occasionally dark brown; clypeus with or without a median tooth (Suppl. material 3: figs S66–S69) ..... **8**
- Body brown to black with an orange tergal maculation on the first gastral tergite; clypeus always with a median tooth (Suppl. material 3: figs S70, S71) .....  
..... *Solenopsis richteri* (alien species not reported from Cyprus)
- 8 Median tooth on clypeus absent; mandibles of major workers with three teeth; occipital margin of head in major workers deeply emarginate; colour variable, from yellow to reddish-brown, occasionally dark brown (Suppl. material 3: figs S66–S67) ..... *Solenopsis geminata* (alien species not reported from Cyprus)
- Median tooth on clypeus always present; mandibles of major workers with four teeth; occipital margin of head in major workers shallowly emarginate; colour uniformly orange/red to rusty (Suppl. material 3: figs S68–S69) .....  
..... *Solenopsis invicta* (alien species not reported from Cyprus)
- 9 Ventrolateral margins of head without longitudinal carina; petiole usually with well-marked peduncle and node (Suppl. material 3: figs S72, S78, S84) ..... **10**
- Ventrolateral margins of head with longitudinal carina; petiole low, without peduncle, gable-like (Suppl. material 3: figs S60, S61) ..... *Myrmecina* (one native species)
- 10 Posterior edge of clypeus raised into sharp ridge in front of antennal insertions (Suppl. material 3: figs S73, S75, S77, S79) ..... **11**
- Posterior edge of clypeus not raised into sharp ridge in front of antennal insertions (Suppl. material 3: figs S95, S97, S101) ..... **22**
- 11 Frontal carinae long, projecting to or behind mid-length of eyes; antennal scrobes present; head in occipital and lateral parts usually with strong reticulate sculpture, never with smooth and shiny areas (Suppl. material 3: figs S73, S75, S77, S79, S81) ..... **12**
- Frontal carinae short, never projecting behind mid-length of eyes; antennal scrobes absent; head usually only with longitudinal sculpture or with smooth and shiny areas (Suppl. material 3: figs S83, S85, S87, S89, S91, S93) ..... **17**
- 12 Propodeum with long spines, longer than width of eye (Suppl. material 3: figs S72, S76, S80) ..... **13**
- Propodeum with short spines, shorter than width of eye (Suppl. material 3: figs S74, S78) ..... **15**
- 13 Dorsum of mesosoma in profile flat to slightly convex with sparse long erect setae (Suppl. material 3: figs S72, S76) ..... **14**
- Dorsum of mesosoma in profile strongly convex with extremely dense long erect setae (Suppl. material 3: figs S80, S81) ... *Tetramorium lanuginosum* (alien species)
- 14 Mandibles finely striated; gaster always darker coloured than head and mesosoma; erect setae between frontal carinae shorter than diameter of eye (Suppl. material 3: figs S72, S73) ..... *Tetramorium bicarinatum* (alien species)
- Mandibles smooth and shiny, only with piliferous pits; gaster the same colour as head and mesosoma; erect setae between frontal carinae mostly longer than diameter of eye (Suppl. material 3: figs S76, S77) .....  
..... *Tetramorium insolens* (alien species not reported from Cyprus)

- 15 Sides of head immediately behind eyes with a single stout projecting hair, directed anteriorly at an angle of about 45.....  
 ..... *Tetramorium delagoense* (alien species not recorded for Cyprus)
- Sides of head immediately behind eyes without such a hair, either hairless or with a number of minute decumbent to appressed hairs ..... **16**
- 16 Frontal carinae strongly developed throughout their length, running unbroken almost to occipital margin; surface of head between frontal carinae strongly granular or reticulate-punctated, appears matt; antennal scrobes well-marked (Suppl. material 3: figs S78, S79) .....  
 ..... *Tetramorium simillimum* (alien species not reported from Cyprus)
- Frontal carinae well developed only to the level of midlength of eyes, behind which they become weak, broken, or gradually fade out posteriorly; surface of head between frontal carinae only finely micropunctated, appears quite shiny; antennal scrobe very shallow, barely marked (Suppl. material 3: figs S74, S75).....  
 ..... *Tetramorium caldarium* (alien species)
- 17 Dorsum of petiole and postpetiole node with strong rugose sculpture, without smooth areas (Suppl. material 3: figs S82, S84) .....  
 ..... *Tetramorium chefketi* and *flavidulum* groups (four native species)
- At least dorsum of petiole with smooth area (Suppl. material 3: figs S86, S88, S90, S92)..... **18**
- 18 Head with complete sculpture of longitudinal rugae ..... **19**
- Head partly with smooth areas.....  
 ..... *Tetramorium semilaeve* group (two native species)
- 19 Gyne with strongly transverse postpetiole at least 2.5 × as wide as long .....  
 ..... *Tetramorium ferox* group (one native species)
- Gyne with not modified postpetiole, at most 1.7 × as wide as long (*Tetramorium caespitum* group; proper species identification needs a complex morphometric procedure and examination of male genitalia) ..... **20**
- 20 Male genitalia: in ventral view one or two corners visible on ventral paramere lobe; in posterior view no distinct emargination between paramere lobes..... **21**
- Male genitalia: in ventral view no corner but rounded ends on ventral paramere lobe; in posterior view distinct division of ventral and dorsal lobe by deep emargination between paramere lobes.....  
 ..... *Tetramorium staerckei* (native species) [Note: separation of members of the *caespitum* group requires combining morphometric data and studies on male genitalia. For more details see keys and photographs in Wagner et al. (2017).]
- 21 Male genitalia: in ventro-posterior view ventral paramere lobe with two corners > 87 µm apart. Worker: generally larger species: CS (arithmetic mean of head length and head width) = 834 ± 56. Usually in anthropogenically influenced, vegetation-free, and even concreted habitats (Suppl. material 3: figs S88, S89)....  
 ..... *Tetramorium immigrans* (alien species)
- Male genitalia: In ventro-posterior view ventral paramere lobe has one corner or two corners, < 87 µm apart. Worker: generally smaller species: CS (arithmetic mean of head length and head width) = 717 ± 52. In both anthropogenic and

- natural habitats (Suppl. material 3: figs S90, S91) .....  
 ..... *Tetramorium indocile* (native species)
- 22 Postpetiole ventrally without tooth or spine (Suppl. material 3: fig. S94) ..... **23**
- Postpetiole ventrally with tooth or spine (Suppl. material 3: fig. S96) .....  
 ..... ***Temnothorax* of the former *muellerianus* group (formerly in the genus *Chalepoxenus*)** (one native social parasite in nests of *Temnothorax*)
- 23 Dorsum of head and mesosoma without standing hairs; postpetiole usually strongly widened, much wider than petiole; propodeum with short, sharp or obtuse denticles (Suppl. material 3: figs S98–S105) ..... **24**
- Dorsum of head and mesosoma with at least sparse, conspicuous standing hairs; postpetiole not strongly widened, only somewhat wider than petiole or even subequal to it; propodeum with spines, denticles, or unarmed (Suppl. material 3: figs S106–S141) ..... **29**
- 24 Very small species, CW < 420 mm .....  
 ..... ***Cardiocondyla minutior*** (alien species not recorded from Cyprus)
- Larger species, CW > 420 mm ..... **25**
- 25 Minute species with CS (arithmetic mean of head length and head width) < 470; head, mesosoma and petiolar segments yellowish-red, red or reddish-brown (Suppl. material 3: figs S98–S101) ..... **26**
- Larger species with CS (arithmetic mean of head length and head width) > 490; head, mesosoma and petiolar segments from brown to black, if mesosoma reddish then head and petiolar segments darker coloured than mesosoma (Suppl. material 3: figs S102–S105) ..... **28**
- 26 Anterior margin of postpetiole deeply emarginate, in anterolateral view with prominent anterolateral corners (Suppl. material 3: figs S100, S101) ..... **27**
- Anterior margin of postpetiole feebly emarginate, in anterolateral view with obtuse anterolateral corners (Suppl. material 3: figs S98, S99) .....  
 ..... ***Cardiocondyla emeryi*** (alien species not reported from Cyprus)
- 27 1<sup>st</sup> gastral segment in a majority of samples with absent or weakly developed blackish pigmentation .....  
 ..... ***Cardiocondyla wroughtonii*** (alien species not reported from Cyprus)
- 1<sup>st</sup> gastral segment in a majority of samples with strongly developed blackish pigmentation ..... ***Cardiocondyla obscurior*** (alien species)
- 28 Postpetiole wide, kidney-shaped; metanotal groove deep; body predominantly or completely black (Suppl. material 3: figs S104, S105) .....  
 ..... ***Cardiocondyla nigra*** (native species)
- Postpetiole moderately wide, oval; metanotal groove shallow; body usually bicoloured with mesosoma paler coloured than head and gaster (Suppl. material 3: figs S102, S103) ..... ***Cardiocondyla mauritanica*** (alien species)
- 29 Median portion of clypeus sharply raised and delineated by a pair of lateral longitudinal carinae (Suppl. material 3: figs S107, S109, S113, S115) ..... **30**
- Median portion of clypeus not raised, evenly convex or somewhat flattened, without carinae or with single central longitudinal carina Suppl. material 3: figs S123, S125, S139, S141) ..... **38**

- 30 Propodeum with short sharp teeth; eyes of workers very small, with less than 20 ommatidia; anterior clypeal margin with two long medial setae; head and mesosoma with strong reticulate and/or rugose sculpture (Suppl. material 3: figs S106, S107) ..... *Stenammas* (one native species)
- Propodeum rounded or at most slightly angled; eyes of workers bigger, with more than 20 ommatidia; anterior clypeal margin with a single long medial seta; head and mesosoma with fine microreticulate or microgranulate sculpture (Suppl. material 3: figs S108–S119) ..... **31**
- 31 Monomorphic species, the largest workers only slightly larger than small workers; antennal scapi long and slim, reaching behind occipital margin of head (Suppl. material 3: figs S108–S113) ..... **32**
- Polymorphic species, major workers with distinctly larger and wider head than in minor workers; antennal scapi short and stout, not reaching to occipital margin of head (Suppl. material 3: figs S114–S119)..... **36**
- 32 First gastral tergite bicoloured; at least partially light-coloured at its basal part (Suppl. material 3: figs S110, S111) ..... **33**
- First gastral tergite dark (brown to black) and uniformly coloured (Suppl. material 3: figs S108, S109, S112, S113) ..... **34**
- 33 2/3 of the first gastral tergite (abdominal segment 4) light-coloured; mesosoma dorsally with erect setae (Suppl. material 3: figs S110, S111).....  
..... *Monomorium pharaonis* (indoor introduced)
- 1/4 of the first gastral tergite (abdominal segment 4) light-coloured; mesosoma dorsally lacking erect setae.....  
..... *Monomorium sahlbergi* (alien species not recorded from Cyprus)
- 34 Whole body uniformly dark coloured, reddish-brown to black, sometimes head darker than mesosoma, and mesosoma brighter than gaster (Suppl. material 3: figs S112, S113) ..... **35**
- Head and mesosoma uniformly coloured, from orange to brick-red, gaster black (Suppl. material 3: figs S108, S109) ..... *Monomorium bicolor* (alien species)
- 35 Body matt and strongly sculptured; body reddish-brown to brown, sometimes head darker than mesosoma, and mesosoma brighter than gaster, but then never with uniform colouration (Suppl. material 3: figs S112, S113).....  
..... *Monomorium subopacum* (native species)
- Body smooth and shiny, body uniformly blackish-brown to black .....  
..... *Monomorium carbonarium* (alien species not recorded from Cyprus)
- 36 Propodeal spiracle vertically slit-shaped or elliptical; anterior clypeal margin with a pair of well-developed strong teeth (Suppl. material 3: figs S118, S119) .....  
..... *Trichomyrmex perplexus* (native species)
- Propodeal spiracle circular or subcircular; anterior clypeal margin without teeth (Suppl. material 3: figs S114–S117) ..... **37**
- 37 Body predominantly dark brown to black (Suppl. material 3: figs S116, S117)...  
..... *Trichomyrmex mayri* (alien species)
- Body predominantly yellow (Suppl. material 3: figs S114, S115) .....  
..... *Trichomyrmex destructor* (alien species)

- 38 Apical club 3-segmented (Suppl. material 3: figs S94, S95, S120–S135); smaller species (1.5 mm) ..... **39**
- Apical club barely marked or 4-segmented (Suppl. material 3: figs S136–S141); larger species (ML > 2.2 mm in the largest workers).....  
 ..... ***Aphaenogaster* (four native species); *Messor* (three native species)**
- 39 Workers dimorphic, head of major workers very large, wider than mesosoma length. Mesosoma of minor workers with deep metanotal groove and antennal scapus longer than head length (Suppl. material 3: figs S120–S135) ..... **40**
- Workers monomorphic; head of workers always shorter than mesosoma length; mesosoma of minor workers without or with shallow metanotal groove (Suppl. material 3: figs S94, S95), if metanotal groove distinct then antennal scapus always shorter than head length (Suppl. material 3: figs S96, S97).... ***Temnothorax*** (12 native species)
- 40 Major workers: head longitudinally striated from clypeus to occiput; occipital lobes mostly with strong longitudinal or reticulate sculpture. Minor workers: mesosoma with distinct microreticulate sculpture, matt or with pronotum mostly smooth and shiny and mesonotum and propodeum with microreticulate sculpture but with shiny background; mesosoma with deep promesonotal groove (Suppl. material 3: figs S120–S127) ..... **41**
- Major workers: head longitudinally striated from clypeus to the middle of head's length; occipital lobes smooth and shiny. Minor workers: mesosoma with pronotum mostly smooth and shiny and mesonotum and propodeum with microreticulate sculpture and shiny background; mesosoma without promesonotal groove (Suppl. material 3: figs S128–S133) ..... **42**
- 41 Very small, length of mesosoma in major workers below 1 mm, in minor workers below 0.6 mm. Major workers: occipital lobes of head and anterolateral corners of pronotum with reticulate sculpture. Minor workers: head and mesosoma mostly with microreticulate sculpture, matt; mesosoma with shallow promesonotal groove (Suppl. material 3: figs S120–S123).....  
 ..... ***Pheidole fadli*** (indoors introduced)
- Larger, length of mesosoma in major workers above 1 mm, in minor workers above 0.6 mm. Major workers: occipital lobes of head with longitudinal sculpture and anterolateral corners of pronotum with reticulate sculpture. Minor workers: head and pronotum mostly smooth and shiny, mesosoma with deep promesonotal groove (Suppl. material 3: figs S124–S127) .... ***Pheidole indica*** (alien species)
- 42 Major and minor workers: propodeal spines minute, not longer than basal width, often in form of small tubercle; postpetiole in profile without conspicuous ventral convexity (Suppl. material 3: figs S128–S131) .....  
 ..... ***Pheidole koshevnikovi*** (native species)
- Major and minor workers: propodeal spines prominent, usually as long as or longer than basal width; postpetiole in profile with conspicuous ventral convexity (Suppl. material 3: figs S132, S133) .....  
 ..... ***Pheidole megacephala*** (alien species not reported from Cyprus)

**Subfamily Ponerinae**

[After Borowiec and Salata (2022) and Schmidt and Shattuck (2014), modified]

- 1 Mandibles with more than 8 dents and denticles, the 1–3 apical dents often somewhat stronger and the following 8–13 dents small to minute; mid and hind tibiae each with one pectinate spur (Suppl. material 3: figs S144–S149) ..... **2**
- Mandibles with 6–7 strong dents of approximately equal size; mid and hind tibiae each with two spurs, median spur large and pectinate, lateral spur much smaller and not pectinate (Suppl. material 3: figs S142, S143) .....  
 ..... ***Cryptopone*** (one native species)
- 2 Ventral apex of the metatibia with a single spur, which is pectinate ..... **4**
- Ventral apex of the metatibia with both a large pectinate spur and a smaller simple spur ..... **3**
- 3 Propodeal spiracle slit-shaped .....  
 ..... ***Parvaponera darwinii*** (alien species not recorded from Cyprus)
- Propodeal spiracle round or ovoid .....  
 ..... ***Brachyponera chinensis*** (alien species not recorded from Cyprus)
- 4 Petiolar base in profile with two small, sharp dents or angles and anteriorly with a circular translucent “window” (Suppl. material 3: figs S144, S145) .....  
 ..... ***Ponera*** (one native species)
- Petiolar base in profile form a simple rounded lobe, without a translucent “window” (Suppl. material 3: figs S146–S149) ..... **5**
- 5 Scape reaching to hind margin of head, scape length/head width ratio > 0.88; petiole in profile significantly higher and narrower; mesopleuron completely covered with carinulate sculpture (Suppl. material 3: figs S146, S147) .....  
 ..... ***Hypoponera eduardi*** (native species)
- Scape not reaching to hind margin of head, scape length/head width ratio < 0.88; petiole in profile significantly lower and thicker; mesopleuron smooth (Suppl. material 3: figs S148, S149) ..... ***Hypoponera punctatissima*** (alien species)

**Discussion**

**Land cover and protected areas**

The number of known alien insect species in Cyprus has increased dramatically during the last decade (Demetriou et al. 2023a); there has been a steep increase in the number of recorded ants in part due to increased sampling intensity (Tables 1, 2). According to our analysis of CLC occupied by alien ants in Cyprus, the majority of species (90%) have been collected from anthropogenic habitats including artificial surfaces and agricultural land, with limited spread to forest and semi-natural areas or inland waters (Fig. 1). These data are in accordance with other similar observations, reporting al-

ien insects, including ants, mostly dominating anthropogenic habitats (Espadaler and Bernal 2003; Lopez-Vaamonde et al. 2010; Schifani 2019; Rosas-Mejía et al. 2021; Demetriou et al. 2023b). Regarding the second level of CLC types, within artificial surfaces more than half of the habitats (54%) correspond to “urban fabric”, followed by “artificial, non-agricultural vegetated areas” (43%). Within agricultural land, “heterogeneous agricultural areas” dominate occupied habitats with a percentage of 69% of obtained records. Lastly, regarding forest and semi-natural areas, half of the occurrence records were classified as “shrub and/or herbaceous vegetation associations” (52%), followed by “forests” (33%) and “open spaces with little or no vegetation” (15%) (Suppl. material 1). Nevertheless, looking in greater detail into these forest and semi-natural areas and comparing these classifications with the notes taken on the habitat during the surveys, some correspond to traffic islands, roadsides, urban habitats and/or other areas with evident human pressure and modifications. This comes to show that the spread of alien ants into natural habitat types is perhaps limited and that their spread is probably favoured by anthropogenic habitat heterogeneity (Elton 1958; Hobbs and Huenneke 1992; Melbourne et al. 2007).

The NATURA 2000 and RAMSAR wetland networks also seem not to be impervious to biological invasions of ants, with seven species being present in a total of 20 protected areas (Suppl. material 2). Many records were situated around the edges of protected areas, as already reported by Liu et al. (2020), with the most noticeable case in Cyprus being that of *W. auropunctata*, collected just outside the protected areas of Faros Kato Paphou (CY4000013), Chersonisos Akama (CY4000010) and Ekboles Potamon Ezousas, Xerou, kai Diarizou (CY4000018) in Paphos. The alien ant fauna of the island both within protected areas and surrounding areas (Holenstein et al. 2021), should be further monitored to increase understanding of potential adverse effects on native biodiversity and protected habitat types. The Akamas Peninsula (CY4000010 and CY4000023) is of particular importance because of increasing pressure from urban developments which may increase the number and spread of alien species. Four alien species, *M. bicolor*, *N. jaegerskioeldi*, *P. longicornis* and *T. lanuginosum* have already been identified from the region, although no adverse effects have been reported. Although protected areas have been reported as refuges for native species against invasive alien species (Gallardo et al. 2017), the spread and establishment of species such as *W. auropunctata* should be monitored further.

## Establishment status and introduction pathways

Twelve out of 17 species are considered to be established with self-sustaining populations on the island; one is considered as an introduction to indoors because it is confined to plant nurseries and the zoo (i.e. *T. bicarinatum*). For four species, *C. cf. vitiosus*, *H. punctatissima*, *P. fadli* and *T. caldarium*, the establishment status is currently unknown because only single records/specimens have been found. These species may represent recent introductions that will fail to establish but ongoing monitoring

is recommended. It is important to mention that the greenhouse in which *P. fadli* was detected, was destroyed and reconstructed (paved) a few months after the specimen's collection (Demetriou pers. obs.).

Although introduction pathways of alien ants in Cyprus are largely unknown, plant nurseries seem to play a crucial role in the movement of alien ants (Jucker et al. 2008; Pospischil 2011; Blatrix et al. 2018). As shown in our study, six alien ant species were collected from plant nurseries and greenhouses with *P. fadli* and *H. punctatissima* known only from such localities. This shows that alien ants can be moved outside their native range in the soil of potted plants, as hypothesised for *W. auropunctata* (Vonshak et al. 2010; Demetriou et al. 2022), potentially bypassing phytosanitary inspections and border controls (Rabitsch 2011; Wong et al. 2023). The identification key aims to support detection and monitoring activities. Quarantine measures or chemical treatment could be implemented for imported plant material to mitigate further spread of invasive alien species such as *S. geminata*, *S. invicta*, *S. richteri*, and *W. auropunctata* (Rabitsch 2022a, b).

## Impacts

There is a lack of information on the environmental and socio-economic impacts of alien ants inhabiting Cyprus, although some invasive alien species have been scored as high through global impact assessments such as *W. auropunctata*, *P. longicornis*, *T. bicarinatum*, and *T. destructor* (Salata et al. 2019; Demetriou et al. 2022; Gruber et al. 2022; present study). Furthermore, according to Salata et al. (2019) aggressive behaviour towards native ant species has been observed by *N. jaegerskioeldi* but such observations are not supported by quantified analyses suggesting the need for further studies. These species should be considered for prioritisation on lists with further ongoing research on their impacts. As an example *W. auropunctata* has been listed both as one of the world's worst invasive alien species (GISD 2023) and as an invasive alien species of Union Concern (EU 1143/2014; EU 2022/1203). Currently, no other native species have been found to occur in sites heavily infested by *W. auropunctata* and for the moment no spread in natural habitats has been observed (Demetriou pers. obs.).

*Wasmannia auropunctata* has the potential to threaten human and animal health because it can sting and in rare cases cause anaphylactic shocks with the potential to harm both wild and domesticated animals (Wetterer and Porter 2003; Kidon et al. 2022). Infestations of electronic devices by *T. destructor* also pose a potentially life-threatening socio-economic impact of ants on the island. Citizen science is being considered as an approach by the authors to assess the socio-economic impacts of ants in Cyprus and particularly their impacts in households. Such an initiative could also be used to enhance understanding of the presence of other alien species, verify past records of *M. pharaonis*, raise awareness of the impacts of alien ants as well as provide adequate data to perform risk assessments under EICAT and SEICAT protocols (Hawkins et al. 2015; Bacher et al. 2018).

## Conclusions

A total of 17 alien ant species have been documented in Cyprus, with nine representing new records for the island. Most species have been predominantly found in urban and agricultural habitats although some observations have been also made in semi-natural and natural habitats including protected areas. All species are largely synanthropic and are distributed in the island's lowlands, with the majority (14 out of 17) being detected within the last decade and considered to be established (12 out of 17) on the island.

Although introduction pathways of alien insects in Cyprus are largely unknown (Demetriou et al. 2023a), the CBD pathway category “Transport – Contaminant: 3.1. Contaminant nursery material” (CBD 2014), is proposed as one of the main introduction pathways of alien ants to the island. Potted ornamental plants seem to facilitate the introduction and spread of nests such as in the case of *W. auropunctata* in Israel (Vonshak and Ionescu-Hirsch 2009).

The identification key aims to support phytosanitary inspections and border controls in order to provide early detection and management of alien ants. Future studies could incorporate molecular methods to assess the biological invasion history and introduction pathways of alien ants to the island. Online, accessible, and more user-friendly identification guides could further enhance monitoring efforts.

Regarding the impacts of alien ants in Cyprus, further monitoring through citizen science initiatives and structured surveys could be insightful particularly for studying the spread of alien ants and their interactions within ecosystems. Data on possible and observed impacts could raise awareness and inform local policy and management actions mitigating the impacts through containment and eradication campaigns.

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## Supplementary material I

### Georeferenced records of alien ant species in Cyprus

Authors: Jakovos Demetriou, Christos Georgiadis, Angeliki F. Martinou, Helen E. Roy, James K. Wetterer, Lech Borowiec, Evan P. Economo, Kostas A. Triantis, Sebastian Salata  
 Data type: csv

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Link: <https://doi.org/10.3897/neobiota.88.106750.suppl1>

## Supplementary material 2

### **Alien ant species in protected NATURA 2000 and RAMSAR areas**

Authors: Jakovos Demetriou, Christos Georgiadis, Angeliki F. Martinou, Helen E. Roy, James K. Wetterer, Lech Borowiec, Evan P. Economo, Kostas A. Triantis, Sebastian Salata

Data type: xlsx

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Link: <https://doi.org/10.3897/neobiota.88.106750.suppl2>

## Supplementary material 3

### **Plates including morphological characteristics used in the identification key to distinguish alien from native worker ants inhabiting Cyprus**

Authors: Jakovos Demetriou, Christos Georgiadis, Angeliki F. Martinou, Helen E. Roy, James K. Wetterer, Lech Borowiec, Evan P. Economo, Kostas A. Triantis, Sebastian Salata

Data type: zip

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Link: <https://doi.org/10.3897/neobiota.88.106750.suppl3>

## Supplementary material 4

### **Description of *Solenopsis* CYP139 and morphometric data for future comparisons**

Authors: Jakovos Demetriou, Christos Georgiadis, Angeliki F. Martinou, Helen E. Roy, James K. Wetterer, Lech Borowiec, Evan P. Economo, Kostas A. Triantis, Sebastian Salata

Data type: pdf

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# Changes in the functional and phylogenetic diversity of above- and below-ground plant communities invaded by two alien herbs

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## Abstract

Introduced plants can have long-lasting and irreversible effects on the communities and ecosystems they invade. A critical step towards understanding the legacy of plant introductions is the characterisation of changes in the invaded plant communities and how these changes are related to biogeochemical modifications. Here, we addressed this issue by comparing the impacts of two large invasive herbs, *Gunnera tinctoria* and *Impatiens glandulifera*, on the compositional, functional, and phylogenetic structure of the standing vegetation (above-ground communities) and the soil seed bank (below-ground communities). The introduction of both invasive species was associated with a significant decrease in above-ground species richness, with subsequent changes in the functional diversity and phylogenetic dispersion of the vegetation. Yet, these invaders differed in their long-term impacts and the reversibility of any modifications they caused. While *G. tinctoria* invasions resulted in phylogenetically clustered communities (both above- and below-ground) that were clearly distinct from uninvaded ones, seed bank communities invaded by *I. glandulifera* were indistinguishable from uninvaded ones, despite major compositional changes above-ground. Further, we found alterations in nutrient cycling associated with *G. tinctoria* invasions that could

facilitate its local persistence and exacerbate any negative effects on native diversity. Our findings suggest a high susceptibility of pre-invasion above-ground communities to colonisation by distantly related herbs. However, the seed banks showed a degree of resilience against both invaders, with no major differences in species richness. Ultimately, differences in the impacts of these large invasive herbs suggest that dominance in the vegetation and a large stature are poor predictors of long-term plant community changes, including regeneration potential from seed, which are associated with plant introductions.

### **Keywords**

Functional diversity, non-native plants, phylogenetic structure, plant invasions, seed persistence, soil legacy, soil seed bank

## **Introduction**

Invasive alien plants represent a significant component of global environmental change (Simberloff et al. 2013; Pyšek et al. 2020) and contribute to the homogenisation of regional floras (McKinney 2004; Castro et al. 2010; Daru et al. 2021b; Yang et al. 2021). Locally, decreases in species richness and changes in the composition of the above-ground vegetation (hereafter vegetation) are among the most visible and frequent impacts of invasions by non-native herbaceous species (Hejda and Pyšek 2009; Pyšek et al. 2012, 2015; Lavoie 2017), especially those that tend to become dominant in the recipient community (Gioria and Osborne 2010; Pyšek et al. 2012; Čuda et al. 2020; Hejda et al. 2021). These changes can have long-term and irreversible effects on ecosystems, altering the regeneration potential of the resident communities through changes in the diversity and composition of the soil seed bank (Gioria and Pyšek 2016) and other soil legacy effects, including modified plant-soil feedbacks and alterations in biogeochemical cycling (e.g., Funk and Vitousek 2007; Ehrenfeld 2010; Yelenik and D'Antonio 2013; Zhang et al. 2022). Early in the invasion, these changes can benefit the invaders themselves or other non-native plants (Simberloff and von Holle 1999; Funk and Vitousek 2007; Yelenik and D'Antonio 2013), with possibly adaptive consequences for both the resident species and the invaders (Xu et al. 2021). The magnitude, direction, and reversibility of these changes will depend, among other factors, on the functional and phylogenetic structure of the resident vegetation, which influences their susceptibility to, or resilience against, invasions (Darwin 1859; Lososová et al. 2015; Hejda et al. 2021; Gioria et al. 2023).

A critical step towards an understanding of the legacy of plant invasions, including local extinctions, is the characterisation of compositional, functional, and phylogenetic changes associated with plant introductions (Drenovsky et al. 2012). This is important not only in the vegetation but also in the soil seed bank (hereafter seed bank) (Gioria and Pyšek 2016). The formation of persistent seed banks is, in fact, a key strategy that plants may adopt to persist in a locality, hedging against the risks of reproductive failure in unpredictable environments (Venable and Brown 1988; Venable 2007) and mitigating any negative effects of unfavourable or suboptimal conditions for germination and growth that are associated with intense competition or other negative biotic interactions, and with natural or anthropogenic drivers (Thompson et al. 1998; Gioria et al. 2020).

Invasive plants can have long-lasting effects on the recipient seed banks through the production of seeds that retain their viability for multiple seasons (persistent seed banks versus transient seed banks; sensu Thompson et al. 1997; Walck et al. 2005) and/or through alterations in the seed banks of the resident species (Gioria et al. 2012; Gioria and Pyšek 2016). Recent evidence based on global data shows that banking on persistent seeds contributes to the establishment and range expansion of naturalised populations and promotes their invasiveness (Gioria et al. 2021). For alien species, spreading mortality risks through time via the formation of persistent seed banks increases the probability of successful recruitment from seed by extending the window of opportunity for successful germination, resulting in repeated episodes of recruitment from the seed bank (Chesson and Warner 1981; Gioria et al. 2021). In grassland ecosystems, these windows of opportunity for the germination of alien species and seedling establishment often coincide with periods when competition for resources with native species is low (Gioria and Pyšek 2017; Gioria et al. 2018). This can facilitate the establishment and expansion of those alien species that are competitively inferior to natives (Gioria and Osborne 2014; Gioria et al. 2021) and is especially important in disturbed ecosystems (Gioria and Osborne 2010).

In terms of the impacts on recipient communities, persistent soil seed banks represent an important component of ecosystem resilience against environmental change (Gioria et al. 2012, 2022; Blossey et al. 2017). Mature adult plants and seeds differ substantially in their metabolic characteristics and ecophysiological requirements (Grubb 1977; Larson and Funk 2016; Carta et al. 2022) so that changes in the biotic and abiotic conditions promoted by an invasive plant can have very different effects above- and below-ground (Gioria et al. 2012; Gioria and Pyšek 2016). This, in turn, will have different effects on the regeneration potential of native communities. The ability to form persistent seeds is especially critical to long-lived resident species reproducing exclusively by seed or those relying on short-distance dispersal for survival and expansion (Gioria et al. 2012). For these species, dispersal through time may be more important than dispersal through space, given that they cannot escape local stochastic phenomena, such as disturbance or negative biotic interactions (Gioria et al. 2021). Thus, the suppression of species with such a strategy due to invasion will have long-lasting implications resulting from the absence of long-lived propagules (Gioria et al. 2012; Gioria and Pyšek 2016). Moreover, since seed banks act as reserves of genetic variability (Templeton and Levin 1979; Honnay et al. 2008), any alteration in their composition can have important implications for the evolutionary response of a species to environmental unpredictability (Venable and Brown 1988; Donohue et al. 2010; Gioria et al. 2020, 2022).

In this study, we addressed this issue by evaluating the potential reversibility of the impacts of two large invasive herbs that are listed as species of Union concern (EU Regulation on Invasive Alien Species 1143/2014), i.e., *Gunnera tinctoria* (Molina) Mirbel (Gunneraceae) and *Impatiens glandulifera* Royle (Balsaminaceae). These species differ in their global significance, with the former having a restricted distribution, due to a preference for high rainfall conditions (Gioria and Osborne 2013), while the latter is highly invasive in many European and North American riparian habitats (Čuda et

al. 2017; Helsen et al. 2021). They share a large stature, high-standing biomass, extensive litter production, and rapid early-season growth but differ in their life history and reproductive strategies (Hejda et al. 2009; Gioria and Osborne 2013; Helsen et al. 2021).

In this study, we focused on evaluating their impacts and to what extent these are short or long-term, depending on whether they involve changes in the compositional, functional, and phylogenetic structure of the above-ground vegetation or the soil seed bank. We also evaluated how these impacts on plant communities might be related to abiotic ecosystem properties. Examination of the functional and phylogenetic changes at the community level will generate insights into whether similarities in functional traits or phylogenetic relatedness among the native and the non-native species play a role in mediating the invasibility of the recipient communities (Darwin 1859; Diez et al. 2008; Divíšek et al. 2018). This, in turn, will be useful in advancing our understanding of the relative importance of environmental filtering versus that of competitive interactions in driving successful establishment, as postulated by Darwin's naturalization hypothesis and the limiting similarity hypothesis; Darwin 1859; Diez et al. 2008; Divíšek et al. 2018), and how random factors might contribute to invasiveness and invasibility (Hejda et al. 2021). Due to the ability of *G. tinctoria* to fix atmospheric N through a unique symbiosis with cyanobacteria of the genus *Nostoc* (Osborne et al. 1991), high net primary productivity (Osborne et al. 1991; Hickey and Osborne 1998), and enhanced litter production, we expected major compositional and functional changes in the vegetation and in the seed bank as well as in ecosystem properties, through changes in the biogeochemical cycling of nutrients. In contrast, based on the findings of previous studies (Diekmann et al. 2016; Čuda et al. 2017), we predicted a higher reversibility of the impacts of *I. glandulifera* both on the seed bank and ecosystem properties, although allelopathic effects on fungal composition and arbuscular mycorrhiza observed in this species have the potential to alter nutrient cycling (Gaggini et al. 2018; Čuda et al. 2020).

## Materials and methods

### Reproduction in the study species

*Gunnera tinctoria* is a perennial herb reproducing both sexually, through the production of thousands of viable seeds per plant, and asexually, via a large rhizomatous system and plant fragmentation (Gioria and Osborne 2013). Under suitable conditions, it can form large and long-term persistent seed banks, in the order of tens of thousands of seeds per square metre (Gioria and Osborne 2010). *Impatiens glandulifera* is an annual herb reproducing exclusively by seeds, with recent evidence showing that at suitable localities, it can form short-term persistent seed banks (Skálová et al. 2019), which can survive longer than previously thought (Beerling and Perrins 1993; Skálová et al. 2019).

## Experimental design and study sites

To evaluate any change in the resident plant communities associated with the invaders, we used a comparative approach contrasting invaded and uninvaded areas, based on the assumption that the ecological conditions and species composition in the former are comparable to those of the uninvaded areas prior to the invasion (Hejda et al. 2009). This approach is typically used in this type of study, since it allows a temporal comparison when information on the pre-invasion conditions is not available, while allowing the collection of an extensive data set in a relatively short period of time. To limit any inherent spatial difference in species composition, soil type, and disturbance regime, we selected closely adjacent invaded and uninvaded areas (Hejda et al. 2009). To maximise the comparability of different areas and to minimise any potential confounding effects, we selected highly invaded sites (>95% vegetation cover of the invaders) that were similar in terms of vegetation history and disturbance regime, using distribution maps of varying age as well as anecdotal information. Based on records by the Botanical Society of Britain and Ireland, both species had been present at the respective study sites since the 1930's (BSBI Online Plant Atlas 2020a, b).

Field data were collected at three sites per invader that are representative of the main ecosystem types colonised by these species in Ireland (Suppl. material 1: table S1). Assessment of the impact of *G. tinctoria* was carried out at three coastal grassland communities located on Achill Island, western Ireland (53°55'19"N, 10°1'29"W), while that of *I. glandulifera* was examined at three riparian grassland communities located along the River Lagan, Northern Ireland (54°33'14"N, 5°55'6"W), using a hierarchical sampling design. Each site was characterised by invaded and comparable uninvaded control areas (50 m<sup>2</sup> each) and five 4-m<sup>2</sup> plots were randomly selected within each of these areas. A census of the standing vegetation within each plot (quadrat) was carried out throughout 2017 and 2018 using the Braun-Blanquet approach, although only 2018 data were used in the analyses for consistency with the seed bank data.

## Soil seed bank assessment

Changes in the seed bank associated with plant invasions were assessed by collecting five replicate soil samples from each of the five plots identified in invaded and uninvaded sites, using soil cores 5 cm in diameter and 10 cm in depth, divided into two depth categories (0–5 cm and 5–10 cm). Soil samples were collected in spring and autumn (2018), after the germination of a substantial portion of the seed bank in the field and after seed dispersal, to evaluate differences in the more persistent and transient components of the seed bank, respectively. In total, we collected 600 soil samples per invader over two sampling seasons.

To estimate the density of viable seeds in the seed bank (per square metre of surface area), we used a modified version of the seedling emergence approach (see Gioria and Osborne 2009a) in which we maintained semi-controlled growing conditions to buffer natural fluctuations in heat, cold and light and expose seeds to a broad range of

temperatures over time (0–29 °C), resembling those recorded in the field. These semi-controlled conditions were achieved by (1) leaving greenhouses unheated during the cold months to provide seeds with a period of cold stratification of a duration similar to that to which they would be exposed in the field, by (2) using natural ventilation during the warm months to avoid exposure of the samples to temperatures that would not be achieved naturally in the field, and by (3) using plastic covers during the warm months to prevent the loss of seedlings due to excessive evapotranspiration. These greenhouses were located at the Rosemount Research Unit (Belfield, Dublin).

The seedling emergence approach was selected because it allows an estimate of the viable portion of the seed bank and enables the identification of the seed flora to species level, although it may fail to detect a portion of the dormant flora whose germination requirements are not met under greenhouse conditions (Thompson and Grime 1979). Soon after collection (in May 2018 for the spring samples and November 2018 for the autumn samples), soil samples were processed before transferring them to pots, and seedling emergence was monitored on a weekly basis for one year for both sets of samples. Soil samples were sieved through a 2-mm mesh to remove plant material, pebbles, and other material and were mixed with sterile John Innes No. 2 potting compost to provide nutrients for germination, improve drainage, and create a favourable substrate for rooting. The mix was spread in a 2-cm layer over a 3-cm layer of sterile sand in 5 cm × 5 cm plastic pots. Commercial muslin was inserted at the base of the pots to prevent loss of sand through the drainage holes. Control pots were filled exclusively with compost over sand and were randomly placed among the sample pots at a ratio of 1:25 to detect and quantify contamination caused by airborne seeds, with their position being randomly changed every four weeks. Pots were filled to pot capacity throughout the duration of the experiment.

Seed persistence was inferred from different assessments: the density of seeds at each sampling depth, with increases in depth indicative of greater seed longevity; the presence of seeds in the seed bank but their absence in the above-ground vegetation (Thompson et al. 1997, 1998); and the timing of seed collection, with samples collected in spring assumed to reflect the more persistent component of the seed bank. The fact that seedling emergence was monitored for one year ensured the exposure of seeds to both periods of cold and warm stratification, thus maximising the probability of germination, although we cannot exclude some seeds entering secondary dormancy due to less than optimal conditions for germination, which may have failed to germinate, or that the conditions for germination or the breaking of dormancy were not met, despite the precautions used in this study.

## Ecosystem properties

Characterisation of the impact of the study invaders on soil properties and  $\beta$ -glucoside enzyme activity was based on analyses of soil samples collected in June and September 2017 at each study site from the same plots where samples for the seed bank had been collected. Within each plot, we obtained three composite samples made up of four

subsamples collected using 3 cm diameter soil cores. Each composite sample was divided into two depths: 0–5 cm and 5–20 cm. Soil cores were extracted between 0–5 and 5–20 cm, using a 3 cm diameter soil corer (for total C% measurements), while a 5 cm diameter corer was used for soil bulk density estimations at each depth. After collection, the soils were homogenised, and subsamples were then used for different chemical analyses, following procedures described in Di Palo and Fornara (2017). Samples were sieved through a 2 mm mesh size and then analysed for total soil C, N, and P concentration, bulk density, and pH. Available P ( $\text{PO}_4^{3-}$ ) was measured using the method of Olsen (Olsen et al. 1982). Samples for C, N, and P concentration were oven dried at 65 °C for four days before being ground to a powder, and their elemental concentration was measured by combustion and gas chromatography (COSTECH Analytical ECS 4010 system). The concentrations of ammonium ( $\text{NH}_4^+$ -N) and nitrate ( $\text{NO}_3^-$ -N) were measured in soil solutions using a Bran-Luebbe AA3 auto analyser (Bran-Luebbe, Mequon, Wisconsin, USA). Soil pH was measured on a 1:5 soil:deionised water suspension. Soil bulk density was measured as the ratio between air-dried soil and soil volume. The activity of 1,4-beta-glucosidase (BG), which is involved in cellulose degradation and can be used as a proxy for soil quality (Dick 1994), was measured using 4-MUB-b-D gluco(pyrano)side as the substrate. A description of the methodology used to evaluate enzyme activity is available in Cenini et al. (2015).

## Statistical analyses

We made rigorous statistical assessments of the changes in the vegetation and the seed bank, separately, by performing multiple analytical approaches that enable an evaluation of compositional changes, changes in the functional composition and the phylogenetic relationships among the species, while accounting for the non-independence caused by a shared evolutionary history among phylogenetically related species (Gioria et al. 2020). To this end, we generated the phylogenetic tree covering all taxa noted in our study from the ‘V.PhyloMaker’ package in R (Jin and Qian 2019), using the `bind.relative` function to attach taxa absent from the implemented mega-tree by Smith and Brown (2018) to their designated genus. Statistical analyses were based on a hierarchical design consisting of five factors: Invader (4 levels); Site (3 levels, nested in Invader); Invasion status (2 levels = invaded versus uninvaded, orthogonal to Invader and Site); Plot (4 levels, nested in Invasion status and Site); and, for the seed bank only, Depth (2 levels = 0–5 cm and 5–10 cm, orthogonal to each factor) and Season (spring versus autumn, orthogonal to each factor), with  $n = 5$  replicates per each combination of factor levels. These analyses were based on data pooled at the plot level, resulting in a total of 40 plots per site, averaging five replicate samples.

To evaluate the impact of each invader on (i) species richness (SR) and (ii) abundance of the standing vegetation (percentage cover) or the seed bank (seedling density per square metre), we performed phylogenetic generalised linear mixed models (pGLMM), in a Bayesian fitting framework, using the R package ‘`phyr`’ (Ives et al. 2022). Species richness (presence-absence) was modelled as a binomial response, while species

abundance was modelled as a continuous linear response. In these models, Invasion status and Invader, and, for the seed bank only, Season, were modelled as fixed effects (predictors). The random component included 7 effects, thus specified in R: the effect of phylogeny (1|species); two random terms, one with phylogenetic covariance matrix and another with non-phylogenetic (identity) matrix (1|species\_\_); the effect of site (1|site); the interaction between Invasion status and the phylogenetic covariance matrix (Invasion status|species); the interaction between invasion condition with phylogenetic covariance matrix and a non-phylogenetic (identity) matrix (Invasion status|species\_\_); and the random effect (1|species\_\_@Site), which excludes the correlations among sites (Ives et al. 2022).

To examine impacts on the phylogenetic structure of the invaded communities, we calculated a range of taxonomic and phylogenetic diversity measures, using the R package ‘picante’ (v. 1.8.2; Kembel et al. 2022): Faith’s phylogenetic diversity (PD), mean nearest taxon distance (MNTD), and mean pairwise distance (MPD). Faith’s PD was measured as the sum of all branch lengths of the phylogenetic tree linking all species in each of the six plant communities (Faith 1992), the mean pairwise distance between all species at each site (MPD) and the mean pairwise distance separating each species at each site from its closest relative (MNTD) (Webb et al. 2002). MPD includes all species pairs, MPD is more sensitive to the basal structure of the tree, while MNTD is more sensitive to the branching toward the tips of the phylogeny (Cadotte and Davies 2016; Mazel et al. 2016). Since PD correlates closely and positively with species richness (SRic, Tucker and Cadotte 2013), we used the package ‘phyloMeasures’ (Tsirogianis and Sandel 2016) to calculate standard effect size of PD (PD<sub>i</sub>), MPD (MPD<sub>i</sub>), and MNTD (MNTD<sub>i</sub>), which were obtained by standardising these measures according to the null model, i.e., the mean and standard deviation (SD) of phylogenetic diversity metrics for a given species richness. This allows comparisons of sites with different SRic (Mazel et al. 2016). These standardised metrics quantify the relative excess (over-dispersion) or deficit (clustering) in phylogenetic diversity for a given species set relatively to the species pool. A negative standardised metric reflects a relative clustering of species while a positive standardised metric reflects a relative over-dispersion of species (Mazel et al. 2016; Tsirogianis and Sandel 2016).

To examine impacts on functional diversity, we calculated six measures that represent the most important dimensions of functional diversity (Mouchet et al. 2010): Functional Richness (FRic), which measures the volume of functional (niche) space filled by the species in a community (abundance is not accounted for); Functional Evenness (FEve), which is the degree to which the biomass of a community is distributed in the niche space; Functional Divergence (FDiv), which is the degree of niche differentiation (Mason et al. 2005); Functional Dispersion (FDis), representing the spread of the species in the trait space (Laliberté and Legendre 2010); and Rao’s quadratic entropy (RaoQ), which is a continuous measure of functional diversity including information about the evenness of the distribution of functional traits within a community’ (Weigelt et al. 2008) and representing a mix between functional richness and functional divergence (Botta-Dukát 2005). Finally, we calculated community-level

weighted means of trait values (CWM; Lavorel et al. 2008), measuring averaged trait values, weighted by the relative abundance of each species, to evaluate whether single traits were affected by dominance by the invaders.

Measures of functional diversity were calculated, either including or excluding the invaders from the analyses, using the dbFD function of the R package 'FD' (v. 1.0-12.1, Laliberté et al. 2022). To calculate these measures of functional diversity, we used six species traits extracted from LEDA (Kleyer et al. 2008), BiolFlor (Klotz et al. 2002), the Seed Information Database (SID) (v. 7.1.; Kew R.B.G. 2022), and GloSSBank (Gioria et al. 2020): life form (annuals versus perennials), adult plant height (H), leaf area (LA), seed mass (SM), seed persistence (P SB), and reproductive strategy (seeders versus resprouters). These traits are known to be relevant to adult plant functions (Díaz et al. 2016) and seed bank properties (Gioria et al. 2021) (Suppl. material 1: table S2).

Ecosystem-level differences were evaluated using 11 variables: soil bulk density (BD), soil pH (pH), total soil C (C), total soil N (N), P, available P (olsen\_P), ammonium ( $\text{NH}_4$ ) and nitrate ( $\text{NO}_3$ ), 1,4-beta-glucosidase (BG), and biomass C (BiomassC). Measures of  $\text{NH}_4$  and  $\text{NO}_3$  at different sampling times were combined as a single variable and used, separately, to analyse the effect on the transient and more persistent component of the seed bank.

Evaluation of the impact on the taxonomic, functional, and phylogenetic diversity of the invaded communities, and changes in ecosystem properties, was made by performing Nonmetric Multidimensional Scaling (NMDS), using the metaMDS function in the R package 'vegan' (v. 2.6-4; Oksanen et al. 2022). Phylogenetic beta diversity was used as the underlying dissimilarity measure and was calculated by computing pairwise and multiple-site phylogenetic dissimilarities using the functions *phylobeta* and *beta* in the R packages 'phyloregion' (v. 1.0.6; Daru et al. 2021a) and *BAT* (Cardoso et al. 2015). To examine changes in the functional and phylogenetic structure of the invaded communities, we used the function *envfit* of the R package 'vegan' (Oksanen et al. 2022), fitting measures of phylogenetic and functional diversity, and single ecosystem (environmental) variables to NMDS axes. For measures of functional diversity, we also performed two-way ANOVA, using the post hoc Tukey's Honest Significant Difference (HSD) test to evaluate the significance of differences between pairs of group means (interaction between Invasion status and plant community component, which is persistent seed bank, transient seed bank, or standing vegetation).

Multivariate analyses were performed using incidence (presence/absence) or abundance data (percentage cover or seedling densities), and either including or excluding the presence and abundance of the invaders from the analyses. Including the invaders allowed us to characterise the new communities created by the invaders and to evaluate the potential contribution of environmental filtering or niche differences in promoting invasion success through a knowledge of whether the invaders are either closely or distantly related to the species in the communities that ultimately become invaded. Conversely, excluding the invaders allowed us to evaluate the changes occurring in the resident species only, and this is especially important where the invaders dominate the vegetation, the seed bank, or both, although the remaining relative abundances

might be strongly affected by spatial patterns (Gioria and Osborne 2010). To make changes in the vegetation and the seed bank comparable in the multivariate space, we transformed both variables using the Wisconsin standardisation. All analyses were conducted in the R software environment (v. 4.2.1, R Development Team 2022).

## Open research statement

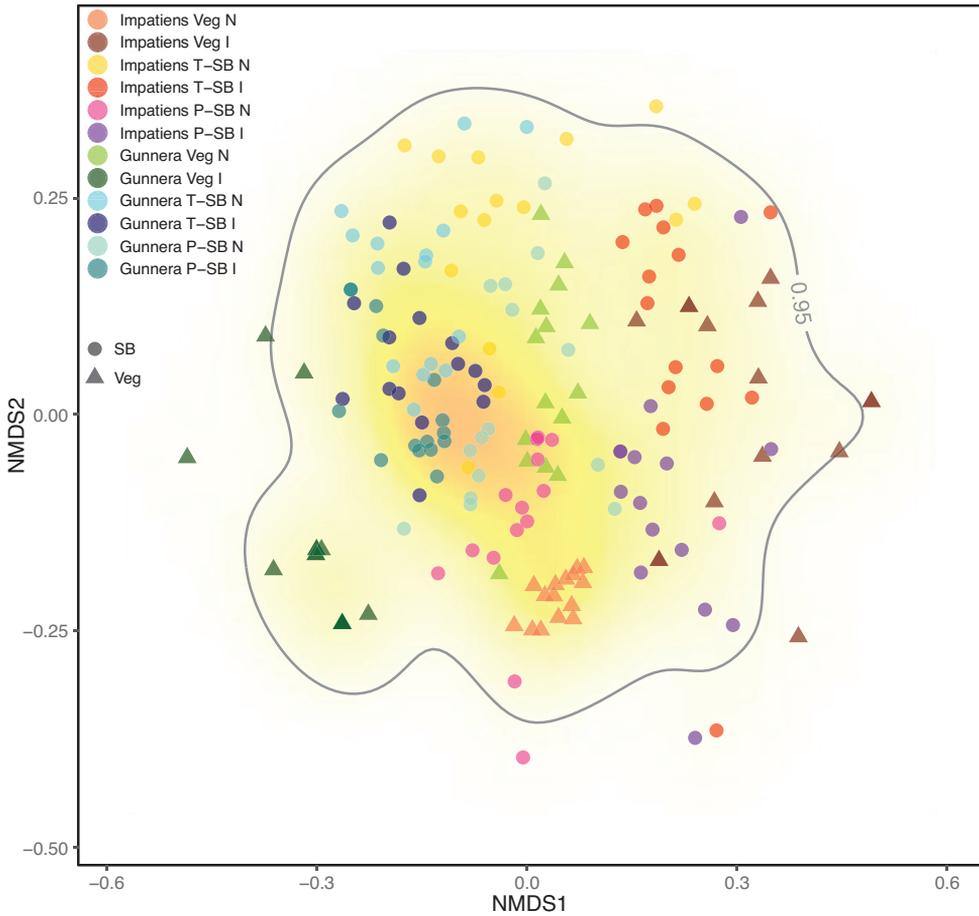
Data on the species recorded in the vegetation and the seed bank and the location where they have been found are presented as supplementary material.

## Results

### Compositional changes

The list of species recorded in the vegetation, with their traits, and the seed banks of invaded and uninvaded areas is presented in Suppl. material 1: tables S1, S2; species that were present only in the vegetation or in the seed bank are also listed in Suppl. material 1: table S1. Plant invasions by either invader resulted in major compositional changes in the standing vegetation, with invaded communities occupying markedly different positions in the multivariate space (Fig. 1) due to the loss of many species (Suppl. material 1: table S1). In total, 12 species were found in the vegetation invaded by *G. tinctoria* compared to 55 species recorded in uninvaded communities, while 13 species were reported in communities invaded by *I. glandulifera* compared to 24 species in uninvaded ones (Suppl. material 1: table S1). Bayesian phylogenetic generalised linear mixed models showed that this reduction in species richness in the invaded communities was significant (estimate = -4.29), with similar effects for both invaders (estimate = -3.93 for *G. tinctoria* and -4.72 for *I. glandulifera*) (Suppl. material 1: table S3). Due to the dominance of the introduced species, the models did not detect any significant difference in mean vegetation cover between invaded and uninvaded communities. When the invaders were excluded, there was a decrease in the cover of the resident species (estimate = -0.30), although the response of the native species was highly species-specific (Suppl. material 1: table S3). Multivariate phylogenetic analyses showed that invaded communities formed separate groups in the ordination plot depending on invader identity (*G. tinctoria* versus *I. glandulifera*), while the uninvaded ones were close to each other, irrespective of whether they were associated with coastal or riparian grasslands, based on either incidence (Fig. 1) or abundance (% cover) data (Fig. 2).

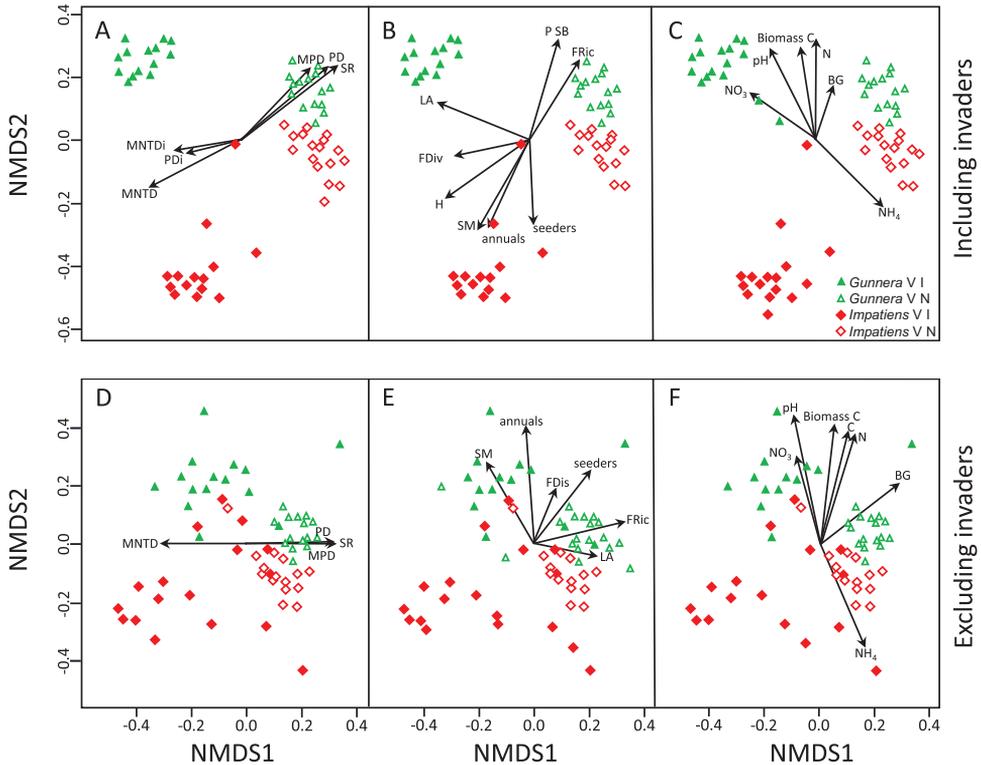
The effects on the composition of the vegetation were more evident than those on the seed bank, especially when the more transient component of the seed bank was considered (Fig. 1). Invaded communities shared the same number of species irrespective of the invader (43 species), while uninvaded adjacent communities supported 47 and 33 species at sites associated with *G. tinctoria* and *I. glandulifera*,



**Figure 1.** Non-metric multidimensional scaling plots representing phylogenetic distances (mean pairwise distance separating each species at each site from its closest relative; MNTD) in the above-ground vegetation (VEG) and the soil seed bank (SB) collected from five comparable invaded (I) and uninvaded (N) plots at each of three sites per invasive species (*Gunnera tinctoria* and *Impatiens glandulifera*), with 95% confidence interval ellipses. The seed bank was collected in the spring and in autumn, reflecting its transient (T) and persistent (P) component.

respectively (Suppl. material 1: table S1). Bayesian phylogenetic generalised linear mixed models did not show any significant difference in species richness for both the transient and more persistent components of the invaded seed banks, irrespective of the invader and whether seedlings of the invaders were included or not (Suppl. material 1: table S4).

Compositionally, both invaders dominated the standing vegetation of the invaded communities, with 100% cover being reached in the summer months, as well as the seed bank. The latter was especially true for *G. tinctoria*, which formed, on average, large and persistent seed banks at each site (mean  $\pm$  S.D. 11,168  $\pm$  3,881



**Figure 2.** Non-metric multidimensional scaling ordinations plotting gradients of phylogenetic distances (**A, D**), functional diversity (**B, E**), and ecosystem properties (**C, F**) above vegetation abundance data (corrected % cover), using MNTD as the underlying distance measure. Each dot corresponds to vegetation data collected from each of five plots within comparable invaded (I) and uninvaded (N) plots at each of three sites per invasive species (*Gunnera tinctoria* and *Impatiens glandulifera*). Analyses both include (**A–C**) and exclude the presence of the invaders (**D–F**).

seedlings per square meter in spring and  $11,718 \pm 3,368$  in autumn), representing ca. 85% of the spring seed bank and ca. 68% of the autumn one. In the uninvaded seed banks, only  $33 (\pm 36)$  seedlings/m<sup>2</sup> were found in spring (the more persistent component of adjacent uninvaded communities), while  $666 (\pm 1,344)$  seedlings/m<sup>2</sup> were found in autumn, reflecting the effect of the most recent seed rain. *Impatiens glandulifera* also formed a viable seed bank that persisted over at least one regeneration season (mean  $264 \pm 188$  seedlings/m<sup>2</sup> in spring and  $376 \pm 168$  in autumn). The contribution of its seeds to the seed bank was, on average, 30.8% in spring and 28.23% in autumn.

Changes in the composition of both the vegetation and the seed bank were, however, not only associated with the dominance of the invaders above- and below-ground, with analyses excluding vegetation cover or seedling densities of the invaders

also revealing major changes in the relative abundances of the resident species and the overall composition of the species assemblage (Figs 1–3). These analyses showed greater similarities within the vegetation of invaded or uninvaded communities, while the vegetation of communities invaded by *G. tinctoria* were moderately distinct from uninvaded ones. The seed banks of areas invaded by *G. tinctoria* were, in contrast, clearly distinct from uninvaded ones, using either incidence or abundance data (Figs 1, 3). Similarly, seed banks invaded by *I. glandulifera* were clearly distinct from uninvaded ones when incidence data were used (Fig. 1) due to the lower richness of these communities, while these differences were less important when seedling densities were used (Fig. 3).

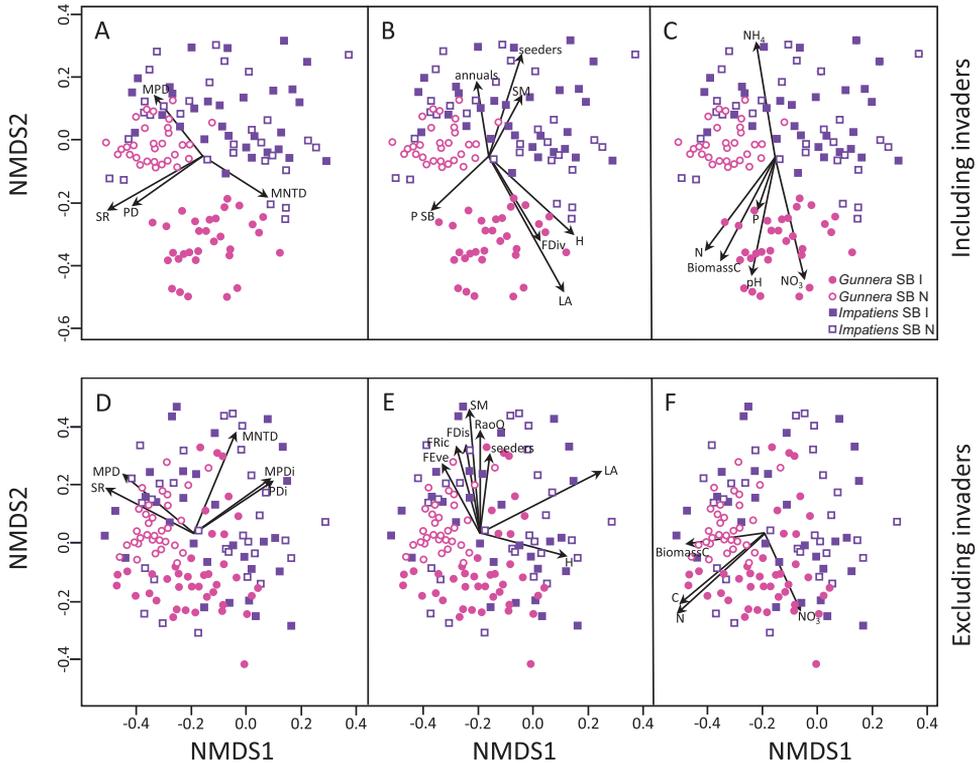
### Changes in the phylogenetic structure of invaded communities

There were major changes in the phylogenetic structure of the invaded communities, which took an opposite direction depending on the invader. In the standing vegetation, analyses including and excluding the invaders showed that uninvaded communities were characterised by a greater PD and MPD, which was associated with a greater species richness, while invaded ones displayed greater MNTD and phylogenetic over-dispersion (Fig. 2A, D). In the seed bank, phylogenetic patterns were less evident, with no clear clustering or over-dispersion. Nevertheless, uninvaded seed banks displayed a generally greater MPD, regardless of the presence or absence of the invaders (Fig. 3A, D).

### Changes in the functional diversity of invaded communities

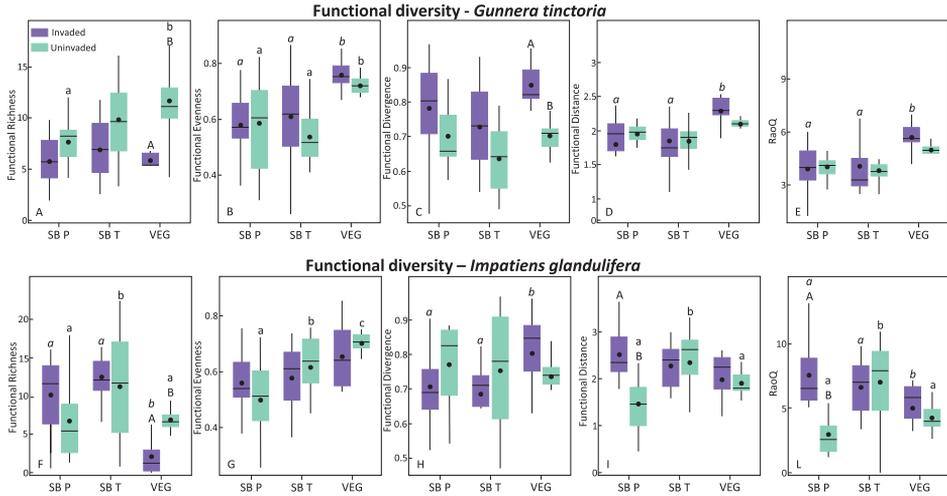
Functionally, the invaded communities were dominated either by a species with a greater leaf area (*G. tinctoria*) or by an annual seeding species with a large seed mass (*I. glandulifera*). However, these patterns were exclusively driven by the presence of the invaders, resulting in a greater functional divergence (Fig. 2B). The uninvaded vegetation was characterised by a greater contribution of seeders, with a greater leaf area, resulting in a greater functional richness and dispersion compared to the invaded one when the cover of the invaders was excluded (Fig. 2E).

The seed banks of invaded or uninvaded sites associated with *G. tinctoria* invasions were composed of a greater proportion of persistent seeds compared to those associated with invasions by *I. glandulifera*. Those sites invaded by *G. tinctoria* were characterised by a distinctly lower functional richness, dispersion, and divergence, and species with a lower seed mass compared to uninvaded ones. In contrast, seeds in both uninvaded and invaded seed banks associated with *G. tinctoria* invasions comprised species with a lower leaf area and plant height than those found at sites associated with invasions by *I. glandulifera* (Fig. 3B). No clear trends were observed in areas invaded by this species, with invaded and uninvaded seed banks being largely indistinguishable (Fig. 3E).



**Figure 3.** Non-metric multidimensional scaling ordinations plotting gradients of phylogenetic distances (**A, D**), functional diversity (**B, E**), and ecosystem properties (**C, F**) above seed bank density data (seedlings per square metre), using MNTD as the underlying distance measure. Each dot corresponds to seed bank data collected twice (spring and autumn), from each of five plots within comparable invaded (I) and uninvaded (N) plots, at each of three sites per invasive species (*Gunnera tinctoria* and *Impatiens glandulifera*). Analyses both include (**A–C**) and exclude the presence of the invaders (**D–F**). Spring and autumn seed banks are displayed as separate plots.

The results addressing functional diversity varied with the index used and the identity of the invader. For both species, functional richness was significantly lower in the invaded vegetation (Fig. 4A, F). For *G. tinctoria*, functional richness was lower in the invaded persistent seed bank compared to the uninvaded one, while the effect of *I. glandulifera* was more complex, with this variable being significantly higher in the persistent component of the invaded seed bank but lower in the invaded vegetation (Fig. 4F). The overall effect of invasion on functional evenness was not significant in either component of the seed bank or the vegetation (Fig. 4B, G). Functional divergence was significantly higher in the vegetation invaded by *G. tinctoria* than in the uninvaded vegetation (Fig. 4C), while differences in uninvaded plots and those invaded by *I. glandulifera* were not significant (Fig. 4H). In areas invaded by *G. tinctoria*, differences in functional distance and RaoQ were not significant (Fig. 4D, E), either



**Figure 4.** Boxplots of five functional diversity indices, based on plant community data collected at three sites for each of two invaders, *Gunnera tinctoria* and *Impatiens glandulifera*. At each site, data were collected from five replicate plots for each invaded (violet) and uninvaded (green) condition. Mean values were plotted as black dots. SB P = persistent seed bank (from samples collected in spring), SB T = transient seed bank (from samples collected in autumn), and VEG (from vegetation surveys conducted over one growing season). The results of post hoc Tukey's HSD tests are displayed on top of the bars: capital letters indicate significant differences between invaded and uninvaded groups, separately for persistent seed banks, transient seed banks, and vegetation. Small letters indicate significant differences between uninvaded groups (persistent seed banks, transient seed banks, and vegetation), while small letters in italics indicate significant differences between invaded groups (persistent seed banks, transient seed banks, and vegetation).

in the seed bank or the standing vegetation, while, again, the persistent component of invaded seed banks was characterised by a significantly lower functional distance and RaoQ (Fig. 4I, L).

### Changes in soil ecosystem properties

The soils of uninvaded communities were characterised by higher  $\text{NH}_4$ , and BG. Areas invaded by *I. glandulifera* were also characterised by higher soil  $\text{NH}_4$  and lower enzyme activities (BG). The soils of areas invaded by *G. tinctoria* were, in contrast, associated with higher  $\text{NO}_3$  concentrations and a higher biomass C (Fig. 2C, F). In terms of differences in the soil seed bank, no clear changes that were related to soil ecosystem properties were identified, except for the seed banks associated *G. tinctoria*, which showed a strong relationship with higher  $\text{NO}_3$ , pH, biomass C, total C, N, and P, as well as with a lower  $\text{NH}_4$ . These patterns were observed either including or excluding seeds of the invader (Fig. 3C, F).

## Discussion

### Compositional, functional, and phylogenetic structure of the vegetation

Our study showed that invasions by two non-native herbs, *Gunnera tinctoria* and *Impatiens glandulifera*, which were phylogenetically distant from the species present in the uninvaded communities, resulted in major compositional and functional changes in the vegetation, suggesting the importance of a low degree in niche overlap, including phenological niches in germination and growth, as a mechanism promoting successful invasions by these species (Webb et al. 2002). Overall, sites invaded by either species had a significantly reduced richness and cover of resident plant species, with associated reductions in functional diversity and changes in the phylogenetic clustering of the recipient vegetation. However, the resulting communities took different trajectories depending on the invader.

Interestingly, the uninvaded above-ground communities (coastal grasslands for *G. tinctoria* and riparian grasslands for *I. glandulifera*) were phylogenetically clustered and shared similar functional traits, despite their habitat differences, while they were compositionally distinct from invaded ones. These communities were characterised by a higher mean pairwise distance (MPD) and increased Faith's phylogenetic diversity, suggesting basal phylogenetic clustering that could be associated with environmental filtering (Webb et al. 2002; Mouquet et al. 2012; Gallien et al. 2014; Mazel et al. 2016). Invaded above-ground communities, in contrast, departed markedly from each other and were phylogenetically over-dispersed, with co-occurring species being more distantly related than in the uninvaded communities, suggesting competitive exclusion among the species co-occurring with the invaders. These communities displayed a greater mean nearest taxon distance (MNTD) and over-dispersion (both including and excluding the invader). This could be associated with intense competition in these communities due to the reduction of available niches following the decrease in functional diversity (Webb et al. 2002; Mouquet et al. 2012; Gallien et al. 2014; Mazel et al. 2016), although more data would be needed to support these conclusions. These results would be consistent with theoretical predictions that non-native species should be sufficiently like native species to overcome environmental filtering but, at the same time, they should be sufficiently different to avoid intense competition (Divíšek et al. 2018), and that phylogenetic over-dispersion is expected in more competitive environments (Webb et al. 2002; Mouquet et al. 2012; O'Dwyer et al. 2012).

From a functional point of view, the invaded above-ground communities were characterised by a lower functional richness than uninvaded communities, reflecting their lower species richness and their higher MNTD. Communities invaded by *G. tinctoria* harboured more annual species and species with a greater seed mass, suggesting that different life form strategies are important to enable coexistence with this dominant invader. Species with a short life cycle tend to rely on long-term persistent seed banks for their survival and persistence in a community under intense competition (Venable 2007). A higher seed mass may, however, confer a competitive advantage at the seedling stage (Turnbull et al. 1999) and allow coexistence with the invader and

the recruitment of these species into the invaded communities, at least in the short term. Invasions by *I. glandulifera*, on the contrary, were associated with low functional diversity and the occurrence of asexually reproducing perennial herbs rather than annuals with more seed-producing species, suggesting a progressive disappearance from the vegetation of plant species that are recruited from the seed bank, especially annual herbs. It is possible that many of the impacts on vegetation are associated with a tendency for both invaders to germinate and/or initiate vegetative growth earlier than most resident species, leading to asymmetric competition and shading effects (as described for e.g., another invader with a similar life form, *Heracleum mantegazzianum* Sommier et Levier: Krinke et al. 2005; Pergl et al. 2007). However, *I. glandulifera* exerted what could be regarded as a positive impact on the invaded communities through the displacement of *H. mantegazzianum* at one site. This provides further evidence that the sites invaded by *I. glandulifera* were already degraded prior to the invasion.

### Compositional, functional, and phylogenetic structure of the seed bank

In terms of seed densities, we observed relatively minor changes in the richness of the soil seed banks of the invaded communities, despite major changes in the composition of the standing vegetation and the almost 100% cover of both invaders. This indicates that seed banks are somewhat resilient against plant invasions, even after several years, regardless of the identity of the invader and the grassland type, confirming the role of persistent seed banks as major components of ecosystem resilience against environmental change (Hopfensperger 2007; Walck et al. 2011; Gioria and Pyšek 2016; Van Looy et al. 2016; Blossey et al. 2017; Gioria et al. 2022). Yet, the composition of the seed bank was much altered by the invaders, suggesting that resilience against invasions (and other environmental changes) might be only temporary and may rely partly on the inputs of propagules from the neighbouring vegetation (Gioria and Pyšek 2016).

Both invaders formed a persistent seed bank, with *G. tinctoria* largely dominating both the more persistent (85%) and transient (68%) components with thousands of viable seeds, a figure comparable to that found previously in coastal areas in this region (Gioria and Osborne 2009b; Gioria et al. 2011). *Impatiens glandulifera* also formed a relatively small but persistent seed bank in the invaded communities, consistent with previous evidence from its invasive range (Skálová et al. 2019). Its seedlings represented nearly a third of the total density of both the more transient and persistent components of the seed bank, suggesting that the production of seeds capable of retaining viability in the soil beyond one season sustains not only the persistence of this species in the invaded communities but also the colonisation of new areas.

Changes in the seed bank differed between the two invaders. Only seed bank communities invaded by *G. tinctoria* were phylogenetically clustered and occupied a clearly distinct niche space. The displacement of resident species from the vegetation through competition and/or the failure of the few species that are present to reach reproductive maturity is likely the major driver of the homogenisation of the seed bank (Gioria and Osborne 2010; Gioria et al. 2011). Functional diversity of the seed bank was higher in

riparian seed banks (both invaded and uninvaded) and was correlated with a greater seed mass and a dominant seeding-only strategy. In contrast, seed banks invaded by *G. tinctoria*, when seedlings of the invader were removed, showed that a lower MNTD corresponded to a lower functional diversity and increasing nitrate pools. Differences in functional diversity between the persistent component of the seed bank and the vegetation, which was observed in most indices for both invaders, further confirms the critical role of seed banks in providing resilience against environmental change (Blossey et al. 2017; Gioria et al. 2022). These findings suggest that ecosystem-level changes promoted by the N-fixing *G. tinctoria* might result, in the longer term, in a loss of closely related species and the persistence of more distantly related species, possibly due to increasing competition for resources in these communities. Based on anecdotal information and  $^{210}\text{Pb}$  and  $^{137}\text{Cs}$  dating data (Fennell et al. 2014), any longer-term impacts would probably be in the order of decades, given that these populations have existed for >70 years.

### Soil ecosystem properties

The precise pre-invasion conditions are unknown and some of the differences between invaded and uninvaded areas might have existed prior to their colonisation by the study alien species. However, the similar changes observed in the multiple sites used in the current study indicate that the modifications are largely invader dependent. Invasions by both species were associated with altered ecosystem properties, but the direction and magnitude of any change depended on the invader. A higher soil nitrate level and pH was found in areas invaded by *G. tinctoria*, although there was no evidence of significant changes in soil C and N stocks, consistent with previous findings (Hickey and Osborne 1998; Mantoani and Osborne 2021). There could also be indirect effects associated with decreases in species richness (and functional diversity) and modifications related to the abundance of the resident species in the vegetation, consistent with experimental evidence for other species (Midolo et al. 2019). Clearly, high seasonal and interannual variability may affect the magnitude and direction of any ecosystem-level impact (Barney et al. 2015). For instance, increases in pH associated with invasions by *G. tinctoria* occur regularly from year to year during the growing season, while values comparable to those in uninvaded areas have been observed during the autumn-winter period (Mantoani and Osborne 2021), suggesting that these changes are mainly seasonal. Although invasions by *G. tinctoria* may not influence soil C and N stocks, this species may alter the availability of inorganic forms of N and is known to have a positive impact on earthworm diversity and abundance (Mantoani et al. 2022).

Changes in pH and nitrate levels and other ecosystem properties associated with *I. glandulifera* invasions were minimal, supporting evidence that the impacts of this invader are, to some extent, reversible (Diekmann et al. 2016; Čuda et al. 2017), although the higher  $\beta$ -glucosidase enzyme activity found in uninvaded areas may indicate some legacy effects. The enzyme  $\beta$ -glucosidase plays an important role in the C and N cycles in soils, and its higher activity is an indication of increased breakdown of cellulose, so more labile C may be available for soil microbes (Stott et al. 2010). However, the enzyme activities were comparable in both uninvaded areas and those invaded by *G. tinctoria* despite

the higher litter inputs (Mantoani et al. 2022). This possibly reflects the greater contribution of earthworms to decomposition processes in invaded areas, where a significant increase in earthworm richness and abundance has been found (Mantoani et al. 2022). A comprehensive assessment of ecosystem-level impacts was beyond the scope of this study and was mainly focused on providing insights into the potential mechanisms underlying the changes in the vegetation and seed bank. Further research on this and whether the changes in pH associated with *G. tinctoria* invasions play a key role in determining the loss of certain species from the vegetation of invaded areas is clearly needed. Overall, there seems to be little correlation between ecosystem properties and plant invasions that can be directly related to the modifications in community composition, suggesting that the limited changes in abiotic factors are largely a consequence of the invasion rather than a major cause of the modifications in the plant communities.

## Conclusion

The introduction of two large invasive herbs into coastal and riparian communities resulted in a reduction in plant species richness and functional diversity, and the phylogenetic clustering of the vegetation. Our examination of compositional and functional changes in the invaded vegetation indicates that these communities are susceptible to phylogenetically and functionally distant non-native species, suggesting that low niche overlap might contribute to the success of these invaders, while competitive exclusion among the species co-occurring with the invaders could be important in shaping the invaded communities. In contrast, the recipient soil seed banks showed some degree of resilience against the impact of invasions, with no major differences in species richness attributable to the two non-native introductions. *Gunnera tinctoria* invasions resulted in longer-lasting alterations through modifications in ecosystem properties in comparison to *I. glandulifera*. In contrast, functional richness and most measures of functional diversity were significantly higher in invaded persistent seed banks than uninvaded ones, confirming the critical function of seed persistence in the soil in providing resilience against environmental changes. Ultimately, this work highlights the difficulties of making generalisations about the ecological impacts of invasive plants even when they share a high stature and a propensity to achieve dominance through asymmetric competition due to early growth and/or germination, and the need for species- and site-specific assessments for developing effective and sustainable control and restoration measures.

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## Supplementary material I

### **Species lists, list of traits, and results of Bayesian phylogenetic generalized linear mixed models of species richness and abundance data in the vegetation and the soil seed bank**

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Data type: xlsx

Explanation note: This supplementary file contains a list of species occurrences in the vegetation and the seed bank at each of six invaded and uninvaded sites. For each species, a list of six species traits is provided. The results of Bayesian phylogenetic generalized linear mixed models of species richness and abundance data in the vegetation and the soil seed bank are also provided.

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Link: <https://doi.org/10.3897/neobiota.88.109185.suppl1>



# Defining the biosecurity risk posed by soil found on sea freight

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## Abstract

Soil associated with sea freight (shipping containers, flat racks and used machinery) arriving at New Zealand seaports was sampled for bacteria, fungi, nematodes, macro-invertebrates and plant seeds. Pseudomonads were selectively isolated, as several significant plant pathogens fall within this bacterial group. The mean and median sample weight collected from sea freight was found to be 417.3 and 152.7 g, respectively, with most recovered soil (73%) collected from the underside of shipping containers and flat rack containers. Likewise, for used machinery, most recovered soil (75%) was found under the machinery. Flat rack containers had significantly higher soil contamination compared to shipping containers and used machinery, but generally the counts and incidence of taxa were significantly lower compared to these other freight types. Viable bacteria, fungi, nematodes, seeds and arthropods were associated with the soil, with both counts g<sup>-1</sup> and prevalence in samples varying with taxa, freight type, and location on the freight. Various regulated biosecurity organisms were recovered from the samples, including *Aphelenchoides besseyi* (rice white tip nematode), and seeds from genera such as *Brachiaria*, *Cortaderia*, *Digitaria*, *Eragrostis* and *Sonchus*. There were also live arthropod taxa that were not recorded as being present in New Zealand. No known plant pathogenic pseudomonads were identified through sequencing of the 16S ribosomal RNA gene. Shipping containers were found to be an important introduction pathway for exotic species, and therefore require careful monitoring and management. Comparisons of the incidence and mean number of organisms associated with soil on sea freight compared to a previous study with soil on footwear, generally showed that incidence and counts of many taxa were lower on sea freight, indicating that biosecurity

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\* Better Border Biosecurity (B3) partner, <https://www.b3nz.org.nz/>.

risk can vary with pathway. However, prioritising one soil pathway over another according to the risks they present, and differentially allocating resources is problematic because the relative risk is dynamic, dictated by factors such as new pests or diseases entering the respective pathways.

### Keywords

Biological invasions, biosecurity, flat rack, invasive alien species, pathway risk factors, pest risk analysis, shipping containers

## Introduction

Globalisation is playing a major role in the spread of invasive alien species (IAS) with profound consequences for environments, economies, and human welfare (Bright 1999; Mack et al. 2000; Early et al. 2016; Diagne et al. 2020). Failure to address biological invasions is expected to result in continued increases in the number of species establishing beyond their native ranges (Levine and D'Antonio 2003; Westphal et al. 2008; Hulme 2009), because establishment rates of IAS show no sign of saturation and those of some taxa may even be increasing (Seebens et al. 2018). International maritime shipping has long been recognised as a pathway for spreading terrestrial IAS including arthropods, vertebrates and plants (Stanaway et al. 2001; Shimono and Konomu 2008; Sardain et al. 2019; Lucardi et al. 2020; Dawson et al. 2022), as well as marine species through biofouling and ballast water (Bax et al. 2003; Briski et al. 2013; Ojaveer et al. 2018; Letschert et al. 2021).

While shipping containers (e.g., typically a 20' twenty-foot equivalent unit (TEU)), or 40' forty-foot equivalent unit (FEU), have increased the volume and efficiency of trade (Reiter 2010), they have also exacerbated the risk of spreading IAS (Catley 1980), including plant pathogens (Gadgil et al. 2000), soil dwelling organisms (Godfrey and Marshall 2002), arthropods (Gadgil et al. 2000; Stanaway et al. 2001; Ward et al. 2006; Fenn-Moltu et al. 2022), reptiles, and amphibians (Kraus 2007; Chapple et al. 2013). The risk posed by soil contaminants on sea freight (e.g., shipping containers, cars, used machinery) is recognised, but is not well understood because IAS in soil are often cryptic and their survival partly depends on environmental factors. Freight arriving at seaports can become contaminated with soil and plant material before shipping, during transport to seaports, while being loaded on ships, and through cross contamination between objects such as stacked containers (Hampson and Wood 1997; Jennings et al. 1997; Hughes et al. 2010). For instance, random sampling of 3681 shipping containers at New Zealand seaports between September 1997 and May 1998, found that 23.4% carried quarantinable contaminants such as soil, viable insects, and plant pathogens (Gadgil et al. 2000).

Environmental exposure can have significant effects on IAS abundance and survival in soil associated with sea containers. For example, a study examining the fate of soil deliberately placed on the roof, under or inside four land-based shipping containers, found that the viability of *Pseudomonas* spp., nematodes and seeds in the soil on

the roof declined over 12 months, with mortality higher for most biota when the soil was exposed to sunlight, moisture or desiccation than when protected (McNeill et al. 2017). Conversely, there were more bacteria and fungi in soil situated in exposed environments, possibly due to ongoing colonization of exposed soil by airborne propagules (McNeill et al. 2017). A study of the microbiota from soil sampled from footwear in passengers' luggage showed high incidences and counts  $\text{g}^{-1}$  of seeds, nematodes, live arthropods, bacteria and fungi, which were comparable to fresh garden soil, probably because they had been protected from potential mortality factors such as extreme temperatures, moisture, desiccation and ultraviolet light (McNeill et al. 2011).

The research reported here examined the biological risk posed by soil on the external surfaces of sea freight (shipping containers, flat rack containers, and used machinery) and compared the results to those from our previous studies on soil biota on contaminated footwear (McNeill et al. 2011) and effects of storage time and environmental exposure on survival of soil biota (McNeill et al. 2017). The outcomes facilitate assessment of relative risks between and within pathways, contributing to the development of recommendations for increasing the efficiency and efficacy of biosecurity interventions.

## Methods

### Soil sample collection and processing

Shipping containers, flat rack containers and used machinery (hereafter collectively referred to as 'sea freight') arriving at two New Zealand seaports, Auckland (36°50.766'S, 174°47.154'E) and Lyttelton (43°36.51'S, 172°43.626'E), were examined and sampled for soil contamination between March and November 2011 and May and June 2012. In New Zealand, biosecurity management of sea freight involves risk profiling prior to arrival. Most freight is free of contamination having been thoroughly cleaned prior to shipping but, where contamination is suspected, freight is visually inspected once it has been offloaded at the port. Therefore, potentially contaminated sea freight was identified prior to arrival through risk profiling by New Zealand's Ministry for Primary Industries (MPI), which is the country's National Plant Protection Organisation (NPPO). Additional contaminated sea freight was identified by port stevedores as it was being unloaded. As the aim of the study was to understand the inherent risk posed by soil contaminants, not incidence of risk, sampling was not random but guided by these processes. Individual shipping containers and flat rack containers were placed on a raised stand with a forklift and all six external sides were visually inspected for contaminants. The location of the contaminants varied but were collected from one or more of the following areas on each container: the roof, outer rail, underside (inner rails or bottom of container), forklift pockets or corner castings. (See Suppl. material 1: fig. S1 for location of collection sites on shipping containers). For flat rack containers, the freight being carried was also inspected. The sides, undersides and tops of used

machinery were inspected, and any contaminants were collected. Soil was collected using either a paint scraper or a plastic hearth shovel and brush, and initially scooped into a sealable plastic container (330 mm L × 330 mm W × 127 mm H) before being transferred to a labelled sealable plastic bag. Where contaminants were collected from more than one location on a container or used machinery, the samples were kept separate. Where practicable, all soil was collected with collection equipment sterilised between samples using 70% ethanol.

Where possible, details were recorded on country of origin of the freight based on the shipping manifest, departure date from the last port of call (which in some cases was different from shipping container country of origin), and arrival date at Auckland or Lyttelton Port. Contamination may not have occurred in the country where the freight originated, as containers may be loaded, unloaded and reloaded at multiple ports prior to arrival in New Zealand. However, as in Gadgil et al. (2000), the last port of loading was used as an indicator of the probable origin of the soil contamination.

Sampling at the ports was carried out in conjunction with biosecurity personnel from MPI. All samples were transferred under a quarantine permit and processing was conducted in PC2 (quarantine) laboratories and glasshouses. Consistent with New Zealand's biosecurity standards, all biological material was destroyed once culturing or rearing, and identification was completed.

In the laboratory, samples were weighed using a bench scale (0–5000 g), then carefully examined under a stereo microscope at a 10–40× magnification to assess the nature of the soil (e.g., rocky, sandy, loess) or coral, and for the presence of biological material (e.g., seeds or arthropods) and other artefacts (e.g., glass, plastic, synthetic fibres).

## Macro-invertebrates

Insects, mites, spiders, arthropod body parts, and molluscs observed under the stereo microscope were removed and counted. When live specimens were found either at the port during sampling or under the stereo microscope, they were collected and killed in 98% ethanol for subsequent identification using the expertise of New Zealand taxonomists.

## Culturable microorganisms

Depending on sample weight, a subsample of between 0.2 (from small volumes of soil) to 21.0 g (for samples over 500 g) was taken to estimate densities (colony forming units per g or CFU g<sup>-1</sup>) of bacteria, fungi, and *Pseudomonas* spp. Each sample was diluted 10-fold using 0.1% peptone and sonicated for 3 minutes to facilitate mixing. Serial dilutions were plated onto three different media: 10% tryptic soy agar plates with 100 mg/L cycloheximide to determine total culturable bacteria counts g<sup>-1</sup> (hereafter referred to as counts); water agar containing 100 mg/L streptomycin for total culturable fungi counts; and Oxoid *Pseudomonas* selective agar (PSA), selective media

for growth and isolation of *Pseudomonas* bacteria supplemented with Oxoid CFC (cetrimide 10 mg/L, fucidin 10 mg/L and cephalosporin 50 mg/L). For total bacteria and *Pseudomonas* spp. isolation, plates were incubated at 20 °C (0:24 h L: D) and colonies counted after seven days. For fungi, plates were incubated at 20–25 °C (0:24 h L: D) and colonies counted after 10 days. For counting, the lowest detection limit was 100 microbial colonies per gram of soil (100 CFU g<sup>-1</sup>). *Pseudomonas* bacteria were chosen as the ‘model’ microorganism for isolation as the genus includes several important plant pathogens (Moore et al. 2006; Silby et al. 2011), with some classified as regulated organisms by MPI.

### **Molecular identification of *Pseudomonas* bacteria**

Sequencing of the 16S ribosomal RNA (rRNA) gene was used to diagnose species from a subset of colonies growing on the *Pseudomonas* selective agar. Colony morphology was visually assessed using characteristics such as colour, shape, and texture, and only the most prevalent colony type, based on its appearance was selected for molecular identification. The dominant colony type for each soil sample was purified by streaking onto nutrient agar and incubated at 20 °C and 0:24 h L: D for three days. Where more than one colony type was prevalent in high numbers for the same soil sample, then up to three morphologically different colonies were selected for further purification onto nutrient agar. A 5 mL nutrient broth culture was inoculated using a single colony from each selected isolate and incubated overnight at 20 °C on a shaker set at 200 rpm. A half mL of each overnight culture was transferred into 0.5 mL of sterile 30% glycerol in a 1.7 mL microcentrifuge tube, sealed with Parafilm M (Bemis Company, Wisconsin USA) and sent to Macrogen Inc. (Seoul, South Korea) for partial 16S rRNA sequencing. Macrogen performed the genomic DNA extraction, amplified the 16S rRNA gene using universal primers 27F and 1492R, then sequenced an internal region of this amplicon using universal primers 518F and 800R.

The sequences were tidied using Geneious version Prime 2021.2 (Kearse et al. 2012) and compared to *Pseudomonas* reference sequences on the Genbank database (Clark et al. 2016) using a BLAST search (Altschul et al. 1997). Multiple alignments of the sea freight isolate sequences and reference species were generated using the program Muscle (Edgar 2004). Visualisation of the genetic diversity between the isolates and reference sequences was conducted in MEGA11 (Tamura et al. 2021). Genetic distance was calculated by the Jukes-Cantor model (Jukes and Cantor 1969) and visualised using a neighbour-joining tree (Saitou and Nei 1987). The robustness of the nodes was assessed with 1000 bootstrap replicates (Felsenstein 1985). Each isolate was assigned to one of three lineages (*P. aeruginosa*, *P. fluorescens* and *P. pertucinogena*) and 14 phylogroups previously described (Mulet et al. 2010; Peix et al. 2018; Passarelli-Araujo et al. 2022). More rigorous methods of diagnosing *Pseudomonas* species using concatenated 16S rRNA and house-keeping genes (Mulet et al. 2010) was beyond the scope of this study.

## Nematodes

For each sample, extraction of nematodes followed the method of Bell and Watson (2001) using the Whitehead and Hemming tray method whereby each sample was placed on two layers of paper tissue (Kimwipes™, Kimberley-Clark Worldwide Inc), supported by two layers of plastic mesh, the top and bottom mesh, 1mm and 44mm square, respectively, which were placed within a shallow tray. The tissue was folded to form an envelope and tap water was added to just cover the envelope. Nematodes were collected from the soil for 72 hours then the liquid was poured into a 1000 mL glass beaker, left to settle for 3–4 hours, and gently reduced to ~75 mL by removing the supernatant. The 75 mL samples were transferred to 100 mL glass Schott bottles and allowed to settle for 3–4 hours before being reduced to a final volume of 20 mL. Nematodes including plant parasitic nematodes (PPN), were counted and identified morphologically using a light microscope at 50× magnification and sorted into feeding groups (e.g., bacterivores, fungivores, predators, omnivores and plant feeders), and where possible genera or family, based on the keys of Siddiqi (2000) for Tylenchida, and Bongers (1994) for other groups. One or two specimens from each feeding group were preserved in a 4% formaldehyde solution containing 1% glycerol and mounted onto slides to enable identification at a 400× magnification. The weight of the subsamples from which nematodes were extracted depended on overall sample size after a subsample had been removed for microbial assessment, and ranged from 0.9 g to 1895 g.

## Molecular identification of nematodes

In addition to samples taken for morphological identification, one or two specimens from each nematode feeding group were also genetically identified. Specimens chosen for DNA sequencing were mostly from taxa within the Tylenchida order, which includes plant parasitic nematodes of biosecurity concern, but specimens from taxa within other feeding groups were also sequenced to confirm morphological identifications. DNA was extracted from single specimens using the prepGEM™ tissue kit (ZyGEM Corporation Ltd., New Zealand) according to the manufacturer's instructions. For all specimens, the primers SSU 18A and 18P (Blaxter et al. 1998), and 1165SR (Ross et al. 2010) were used to target a portion of 18S small subunit rDNA gene. For spiral nematodes, the D2A and D3B primers (Subbotin et al. 2007) were used to target the D2-D3 region of the large subunit rDNA. DNA was amplified in 25µl reactions using 1× buffer (Thermo Scientific Finnzymes), 0.2 mM dNTPs, 0.3 µM of each primer, 0.2 mg/mL BSA and 0.5 units of Phusion Hot Start II Hi-Fi DNA polymerase (Thermo Scientific Finnzymes). Thermo cycling included an initial denaturing at 98 °C for 2 minutes, then for 40 cycles, 98 °C for 10 seconds, 57 °C for 30 seconds, 72 °C for 60 seconds, with a final extension step of 72 °C for 5 minutes. The product was purified using the EZNA Cycle-Pure kit V-spin (Omega Bio-tek). Fragments were sequenced by Massey Genome Service (Massey University, New Zealand) and cleaned using the computer programme Sequencher 4.6 (Gene Codes Corporation, USA) and Geneious 10.0.9 (Kearse et al. 2012). Sequences were compared to nematode sequences on the Genbank database (Clark et al. 2016) using a BLAST search (Altschul et al. 1997).

## Seeds

Seeds were recovered during the initial laboratory inspection of samples under a binocular microscope, counted, and forwarded to the New Zealand National Seed Laboratory for identification and viability testing. Most seeds were identified to at least genus. The plant type (e.g., grass, herb, tree) to which the seed belonged was classified using those in 'Flora of New Zealand' (Webb et al. 1988; Edgar and Connor 2000). Viability was assessed either by dissecting the seed to observe the cotyledon, or by squashing the seed to determine if it had a milky white consistency indicating a healthy endosperm.

Because visual searches are imperfect at detecting all seeds in soil samples (e.g., McNeill et al. 2011), soil used for nematode extraction (described above) was kept allowing viable seeds to germinate. Small (< 100 g) subsamples were spread in a 5 mm thick layer on a paper towel over moist potting mix. Larger subsamples were spread on a layer of moist paper towels in a small tray. The soil was kept moist under natural light in a PC2 glasshouse (15–35 °C) and checked 1–3 times per week for seedlings, which were counted and if necessary transplanted into sterile potting mix 1–2 days after emergence and allowed to grow further for identification. When plants flowered, voucher specimens were collected and transferred to the Allan Herbarium PC2 specimen reception (Landcare Research, Canterbury Agriculture & Science Centre, Lincoln, New Zealand). Identifications were made using web-based keys (e.g., <http://www.efloras.org/index.aspx>) and publications relevant to the origin of each subsample (e.g., Whistler 1995). For each subsample, seeds recovered during visual searches and seedlings observed in germination tests were counted and adjusted to seeds per gram of soil.

## Molecular identification of seeds

For plants in which the morphological identification was uncertain, DNA was extracted from leaf tissue using a Qiagen DNeasy Plant Mini kit (Qiagen Ltd., New Zealand) following the manufacturer's instructions. DNA samples were PCR amplified and sequenced for the following chloroplast and ribosomal nuclear regions: two internal transcribed spacers (ITS1 and ITS2) that flank the 5.8 S nuclear ribosomal DNA region (White et al. 1990); chloroplast *trnL-trnF* spacer region (Sang et al. 1997); 600 bp or 1200 bp of the chloroplast *rbcL* gene (Levin et al. 2003; Kress and Erickson 2007); chloroplast *ndhF* gene (Olmstead and Sweere 1994); intron region of a chloroplast tRNA gene (*trnL*) (Taberlet et al. 1991); and the external transcribed spacer (ETS) of the 18S–26S nuclear ribosomal DNA (Wright et al. 2001). The primers used for each region were those given in the publications cited. Any new-to-New Zealand plants were identified using a combination of morphological keys and genetic methods (James et al. 2014). Edited DNA sequences were compared against sequences from the GenBank database (Clark et al. 2016) using a BLAST search (Altschul et al. 1997).

## Determining regulated taxa

To determine if taxa identified in our soil samples could be regarded by NPPOs as a biosecurity risk, we searched the online Pest Register maintained by MPI

(<https://pierpestregister.mpi.govt.nz/>). This register includes information on pests and pathogens that can affect plant, animal or human health in New Zealand, including unwanted, notifiable, regulated and non-regulated organisms. Anything not listed on the register returns a 'no-record' response.

## Data analysis

Relationships were analysed between various predictor variables (sample weight, freight type, sample origin, sample age, departure season) and response variables (counts of bacteria, fungi and *Pseudomonas*, and both counts and prevalence of nematodes, PPN and seeds). Prevalence (%) was determined as the percentage of samples containing each organism. Three different statistical programs were used, depending on the characteristics of data being analysed, with SAS used for negative binomial generalised linear models (GLMs) (SAS Institute Inc. 2011), R for zero-inflated count models (R Core Team 2019), and Minitab for binomial GLMs (Minitab Inc. 2010). Analyses of macro-invertebrate counts and prevalence were not performed because of extremely low prevalence in samples, and the very similar and small numbers recovered per sample could not provide any meaningful results for interpretation.

## Predictor variables

Sample weights were treated in two ways for separate independent analyses: by classifying them into five classes (0–20g, 21–100g, 101–300g, 301–600g and 601–5000 g) for analysis as a factor; and by  $\log_e$ -transformation for analysis as a covariate. This is because despite sample weight being a continuous predictor, it is possible that its effect is not necessarily continuous. The sample weight class variable allowed the detection of non-continuous sample weight effects (e.g., only the largest weight class has a significant effect on a response variable while other weight classes do not).

Freight type was analysed as a factor with three levels: shipping container, flat rack container, or used machinery. Sample origin was independently analysed using three sets of classifications: country of origin of the sea freight, based on the shipping manifest (six specific countries, hence six levels). EcoRegion as defined by Terrestrial Ecoregions of the World (Oceania, Australasia, Nearctic, Neotropic, Palearctic, or Indo-Malay; there was no sample from the Afrotropic region) (Olson et al. 2001), and UNRegion as defined by United Nations geoscheme region (Asia, The Americas, Europe, or Oceania; there were no samples from Africa or the Middle East).

Sample age was roughly estimated as the date sampled at the New Zealand port minus the date of departure from the last port of call (days) and treated as a numerical variable.

Departure seasons were broadly classified into 'autumn' for freight that departed during February–April and 'winter' for June departures and 'spring' for November departures, since all last ports of call were in the Southern Hemisphere.

## Response variables

Counts of bacteria, fungi and *Pseudomonas* were analysed using a generalised linear model (GLM) assuming negative binomial distributions through a log link function. The negative binomial GLMs were chosen because counts showed over-dispersion, which was beyond the level modelled by Poisson GLMs. The model included freight type for comparison and the  $\log_e$ -transformed sample weight covariate to adjust for weight differences among samples. The effects of the remaining predictor variables were then evaluated by stepwise inclusion, and this process was repeated by replacing the  $\log_e$ -transformed sample weight covariate with sample weight class factor. Interactions between the predictors were not included in the models. In each stepwise inclusion process, a predictor variable was added only if the variable explained a statistically significant amount of deviance assessed by its Chi-square statistic – that is, only if the significance level of the deviance was smaller than or equal to 0.05.

Counts of nematodes (total nematodes and PPN) and seeds were analysed using zero-inflated count models, which were a conditional combination of two GLMs: a GLM assuming a binomial distribution through a logit link function; and on the conditional to the binomial GLM, a GLM assuming negative binomial distribution through a log link function. The zero-inflated count model analysis was required to account for a large proportion of zero counts, which were not explained by a negative binomial GLM alone. Negative binomial models were used because these counts showed over-dispersion when the counts were not zero. For each variable, the zero-inflated count model analysis proceeded in the same way as for the bacterial, fungal and *Pseudomonas* counts by examining freight type effects first, which were adjusted using the  $\log_e$ -transformed sample weight covariate, followed by stepwise inclusion of other factors/variables. Interactions between the predictors were not included in the models. Then, the whole process was repeated by replacing the sample weight covariate with the categorical weight variable.

Prevalence of nematodes and seeds (percentage of samples in which each taxon was found) were analysed, using a GLM assuming binomial distributions through a logit link function. The binomial GLMs were appropriate for modelling a binary event, such as presence/absence of taxa. For prevalence analysis, freight type effects (adjusted for sample weight differences) were examined first, followed by stepwise inclusion of other factors/variables. Analysis was repeated using the sample weight factor term. Interactions between the predictors were not included in the models.

## Position of soil on shipping containers

A separate set of analyses was conducted on counts and prevalence of different organisms in relation to position of samples on shipping containers. Position had four levels: roof, outer rail, underside and fix points. Fix points included forklift pockets and corner castings because the numbers of samples from these positions were small and their descriptive statistics suggested no apparent differences in sample weight or

organism counts and prevalence. The effect of position was examined first using the  $\log_e$ -transformed sample weight covariate for weight difference adjustment. This was followed by testing the effects of other predictors in the same stepwise process as described previously, then, repeated again by replacing the sample weight covariate with the weight factor. Negative binomial GLMs were used for counts of bacteria, fungi and *Pseudomonas*, zero-inflated negative-binomial count models were for counts of nematodes and seeds, and binomial GLMs were for prevalence of nematodes and seeds. These models were chosen due to the same reasons as described previously.

## Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

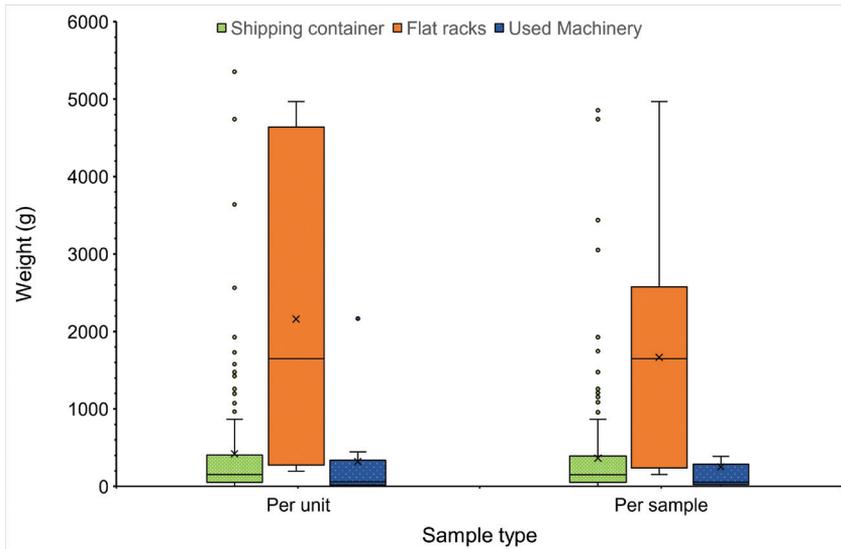
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## Results

### Soil collection and composition

A total of 191 soil samples were obtained from 145 shipping containers (168 samples), seven flat rack containers (9 samples) and 11 items of used machinery (14 samples) (Suppl. material 2: table S1). Used machinery included vacuum tankers, all-terrain vehicles, an excavator, a caravan and forklift tines placed on a flat rack. We also inspected shipping containers that had been profiled prior to arrival (including from Asia and North America) but were found to be clean. Flat rack containers had significantly higher ( $p < 0.001$ ) mean soil weights ( $2161.4 \pm 756.65$  g) than shipping containers ( $422.0 \pm 65.98$  g) and used machinery ( $321.1 \pm 189.98$  g). Summaries of sample weights by freight type are presented in Fig. 1. For all analyses using  $\log_e$ -transformed sample weight covariate, the overall median weight of 152.7 g was used for estimating the mean and standard error of the analysed count/prevalence.



**Figure 1.** Boxplot indicating the amount of soil (g) (minimum, first quartile, median, mean, third quartile, and maximum) collected from shipping containers, used machinery and flat rack containers at New Zealand seaports per unit (individual freight or machinery item) and per sample, respectively.

Over 90% of shipping containers and flat racks (142/152) sampled originated from Australia ( $n = 36$ , 24%) and the Pacific Islands, with most island samples originating from Tonga ( $n = 59$ , 39%) and Vanuatu ( $n = 21$ , 14%) (Suppl. material 2: table S2). Used machinery originated predominantly from Australia (55%,  $n = 6$ ), with the remaining five pieces from England, Wallis and Futuna Islands, Italy and USA.

Sixty-two percent of samples from shipping containers (104/168) were collected from under surfaces, mainly inner rails. The remaining samples were collected from corner castings ( $n = 8$ ), forklift pockets ( $n = 12$ ), outer rails ( $n = 36$ ), roofs ( $n = 7$ ), and the power unit bay of a refrigerated container ( $n = 1$ ). Similarly, 78% of samples from flat rack containers (7/9) were from the underside, and the remaining two samples were from the container deck. On six occasions, soil was collected from multiple positions on shipping containers at the forklift pockets, underside (from both inner rails and container bottom) or outer rail. Samples collected from each container were kept separate for analysis. With used machinery, 64% of samples (9/14) were from vehicle undersides, with the remainder from the top of an excavator track ( $n = 1$ ) and its associated stump-grinding head ( $n = 1$ ), forklift tines ( $n = 2$ ), and the upper cavities of a telehandler stabiliser points ( $n = 1$ ).

All but one of 191 samples comprised a mixture of soil, compost, plant litter, plant debris, and non-organic matter components such as microfine to very coarse rocks (width range <math>1\text{--}20\text{ mm}</math>), crushed coral and sand; the other sample consisted of bark pulp. Plant material in the samples comprised seeds, leaf material, plant fibres, woody fragments, and moss, either solely or mixed. The condition of grass leaves ranged from fresh to dry or senescing. Other organic and inorganic material in the samples included seashells, feathers, animal fibres, plastic, glass, metal, paint fragments and synthetic fibres.

## Macro-invertebrates

Thirty eight of 191 samples (20%) contained live individuals, cadavers and/or body parts of insects, spiders, mites, a millipede and a springtail. Macroinvertebrates were found in soil collected from 27 shipping containers, three flat racks and four items of used machinery. Seven (4%) contained live specimens all recovered from shipping containers, and comprised a small range of taxa including a spider, mite, ants and parasitoid wasps (Suppl. material 2: table S3). No other taxa (e.g., annelids, molluscs) were found.

## Culturable microorganisms

Bacteria, fungi and *Pseudomonas* were present in most samples (82–99%). Counts of bacteria and fungi were obtained from 184 of 191 samples. Due to both the spreading morphology of some bacteria and fungal overgrowth, accurate counts could not be obtained from some samples, and these were excluded. Bacteria were cultured from 181 of 184 samples (98%), with a mean (range) of  $2.16 \times 10^7$  ( $0-7.24 \times 10^8$ ) CFU g<sup>-1</sup> soil. *Pseudomonas* bacteria were isolated from 151 of 184 samples (82%) with a mean (range) of  $8.83 \times 10^4$  ( $0-3.71 \times 10^6$ ) CFU g<sup>-1</sup> of soil. Fungi were present in 182 of 184 samples (99%) with a mean (range) of  $1.44 \times 10^5$  ( $0-4.09 \times 10^6$ ) CFU g<sup>-1</sup> of soil.

## Molecular identification of *Pseudomonas* bacteria

Just under half (70/152) of the soil isolates which produced colonies on PSA either yielded bacterial growth that contaminated the plates or yielded low numbers of colonies and were excluded from our investigation of the dominant *Pseudomonas* spp. A total of 119 isolates were used in the molecular identification of *Pseudomonas* species. Identical sequences that arose from morphological variants isolated from the same soil sample (7), and sequences outside of the Family Pseudomonadaceae (35), were removed from the analysis. The remaining 16S rRNA isolate gene sequences (77) were deposited with GenBank under the accession numbers listed in Suppl. material 2: table S4. Most *Pseudomonas* isolates (83%) came from shipping containers, with Tonga representing the most common country of origin (40%) (Suppl. material 2: table S4). Distance analysis of the 16S rRNA isolate sequences (Suppl. material 1: fig. S2) with reference species (Suppl. material 2: table S5) aided assignment of the isolates into described lineages and phylogroups (Suppl. material 2: table S5; Mulet et al. 2010; Peix et al. 2018; Passarelli-Araujo et al. 2022). All three *Pseudomonas* lineages (*P. aeruginosa*, *P. fluorescens* and *P. pertucinogena*) and 9/14 of the described phylogroups (Mulet et al. 2010; Peix et al. 2018; Passarelli-Araujo et al. 2022) occurred in the isolates (Suppl. material 2: table S4). Almost 64% (49/77) of isolates showed sequence similarity to the *P. aeruginosa* lineage, 35% (27/77) were most similar to the *P. fluorescens* lineage, and the remaining isolate (1/77) aligned with the *P. pertucinogena* lineage. Within the *P. aeruginosa* and *P. fluorescens* lineages, the most common phylogroups were *P. stutzeri* (80%, 39/49) and *P. putida* (70%,

19/27), respectively. No relationship was observed between country of origin and *Pseudomonas* species.

## Nematodes and molecular identification

Most (187/191, 98%) samples were large enough to screen for nematodes after microbes had been extracted. Three samples contained only a small amount of soil which meant that there was only enough for microbial extraction. The fourth sample was discarded due to a processing error during setup. Of 187 samples, 38% (72/187) contained nematodes. Total counts from the 72 samples ranged from 1 to 2130, with mean and median counts of 2.4 and 0.3 g<sup>-1</sup> of soil, respectively. Bacterivores were the most diverse and numerous, followed by predators (Table 1). About half of samples with nematodes included genera classified as potential plant feeders such as *Aphelenchoides* (28), *Aphelenchus* (4) and *Ditylenchus* (14) (Table 1). Tylenchidae (10), which contains species associated with plant damage (Siddiqi 2000), were also present (Table 1). Sequencing results indicated nematode species included *Helicotylenchus dihystera* (Cobb) Sher; *Ditylenchus dipsaci* (Kühn) Filipjev (stem nematode); *Aphelenchoides besseyi* Christie (rice white tip nematode) or *A. fujianensis* Zhuo, Cui, Ye, Luo, Wang, Hu & Liao; *A. bicaudatus* (Imamura) Filipjev & Schuurmans Stekhoven; *A. fujianensis*; *Aphelenchus avenae* Bastion, and *Neopsilenchus magnidens* (Table 2).

**Table 1.** Incidence and number of live nematodes extracted from 72 soil samples removed from sea freight arriving at Auckland and Lyttelton sea ports. A total of 187 samples were used for nematode extraction.

Nematode taxa	Incidence in samples	Mean nos/ sample	Range
<b>Bacterivores</b>			
Cephalobidae	29	75	1–1160
Monohysteridae	2	16	14 & 18
<i>Panagrolaimidae</i>	48	143	1–1890
Plectidae	23	40	1–251
<i>Prismatalaimus</i>	1	16	–
Rhabditidae <sup>a</sup>	17	11	1–53
<i>Rhabdolaimidae</i>	2	9	1 & 16
<b>Fungivores</b>			
<i>Aphelenchoides</i> <sup>a</sup>	28	44	1–420
<i>Aphelenchus</i> <sup>a</sup>	3	8	2–13
<b>Predators</b>			
Diplogasteridae	1	10	–
Monochidae	1	8	–
Tobrilidae	2	17	2 & 32
Tripylidae <sup>c</sup>	3	32	2–84
<b>Omnivores</b>	20	29	1–201
<b>Plant feeders</b>			
<i>Helicotylenchus</i> <sup>a</sup>	2	1.5	1–2
<i>Ditylenchus</i> <sup>ab</sup>	14	10	1–30
Tylenchidae <sup>ad</sup>	10	59	1–500

<sup>a</sup> Taxa contains species regulated by New Zealand Ministry for Primary Industry (01 June 2023).

<sup>b</sup> Includes five samples in which a DNA match could not distinguish between *Anguina*, *Ditylenchus* or *Subanguina*.

<sup>c</sup> Separating Tobrilidae and Tripylidae morphologically is difficult.

<sup>d</sup> Excludes *Ditylenchus* and *Helicotylenchus*.

**Table 2.** Nematode species that were identified by molecular identification. Nematodes recovered from soil removed from sea freight arriving at Auckland and Lyttelton sea ports. Sequences that had 98% or better alignment to those listed on the BLAST website were considered to be a good match.

Possible plant feeders	Number of samples	Number sequenced	Results*
<i>Helicotylenchus</i>	2	3	3 <i>H. dihystera</i> (99%)
<i>Ditylenchus</i>	14	13	2 <i>D. dipsaci</i> (99%) 2 <i>D. dipsaci</i> (96–97%) 4 <i>Ditylenchus</i> sp. (<98%) 5 <i>Anguina</i> or <i>Ditylenchus</i> or <i>Subanguina</i> <sup>b</sup> (<97%)
<i>Aphelenchooides</i>	28	26	5 <i>A. besseyi</i> <sup>a</sup> / <i>A. fujianensis</i> (99%) <sup>b</sup> 1 <i>A. bicaudatus</i> (99%) <sup>a</sup> 1 <i>A. bicaudatus</i> (96%) 2 <i>A. fujianensis</i> (99%) 1 <i>A. saporophilus</i> <sup>a</sup> (96%) 16 <i>Aphelenchooides</i> sp. (<96%)
<i>Aphelenchus</i>	3	4	4 <i>Aphelenchus avenae</i> (99+%)
Tylenchidae	10	7	1 <i>Neosilenchus magnidens</i> (99%) 6 <i>Filenchus</i> sp. (<98%)

\* n refers to number matching the nematode species and % alignment;

<sup>a</sup> Regulated by New Zealand Ministry for Primary Industry (01 June 2023);

<sup>b</sup> Blast match unable to distinguish between these genera for these samples;

Note: Tylenchidae “Associates of algae, mosses, lichens and plant roots, but generally not root parasites of any significance” (Siddiqi 2000). *Ditylenchus* “Fungal feeders and parasites of higher plants, several species, including the type species, are capable of attacking aerial parts.” (Siddiqi 2000).

## Seeds and molecular identification

Seeds were present in 28% of samples (52/187), with a mean of 0.03 seeds g<sup>-1</sup> soil across all samples, and a median and maximum of 0.00 and 1.7 seeds g<sup>-1</sup> soil, respectively. Samples with seeds had a mean of 0.13 seeds g<sup>-1</sup> soil with a median of 0.02 g<sup>-1</sup> soil. Grass species predominated (61%, 589/974), followed by herbs (20%, 197/974) (Table 3), with 15 and 21 genera, respectively. DNA sequencing and morphological examination diagnosed 11 plant species (Suppl. material 2: table S6), of which seven were new records to New Zealand. These were *Ludwigia octovalvis* (Jacq.) Raven, *Hyp-tis pectinata* (L.) Poit., *Eriochloa procer*a (Retz.) C. E. Hubb., *Fimbristylis cymosa* R. Br. var. *cymosa*, *Tribolium oblitterum* (Hemsl.) Renvoize, *Euphorbia prostrata* Aiton, and *Cyperus iria* L.. All sequences that could be taxonomically assigned were deposited in GenBank (Suppl. material 2: table S6).

## Comparing types of sea freight

There were no significant differences in bacteria counts in soil collected from shipping containers, flat rack containers and used machinery (Table 4). Mean counts of both fungi and *Pseudomonas* spp. were significantly higher in samples from shipping containers and used machinery than from flat rack containers (fungi,  $p < 0.001$ , Chi-square = 15.3, df = 2; *Pseudomonas*,  $p < 0.001$ , Chi-square = 37.5, df = 2). Counts of *Pseudomonas* spp. were significantly higher in used machinery than in shipping containers ( $p < 0.001$ ).

**Table 3.** Plant taxa represented, numbers and viability of seeds recovered from soil removed from sea freight arriving at Auckland and Lyttelton sea ports.

Plant type	Total seeds	% viable seeds	Genus
Grass	589	70.6	<i>Axonopus</i> , <i>Brachiaria</i> <sup>a</sup> , <i>Chloris</i> <sup>a</sup> , <i>Cortaderia</i> <sup>a</sup> , <i>Digitaria</i> <sup>a</sup> , <i>Echinochloa</i> <sup>a</sup> , <i>Eleusine</i> , <i>Eragrostis</i> <sup>a</sup> , <i>Eriochloa</i> , <i>Megathyrsus</i> , <i>Panicum</i> <sup>a</sup> , <i>Paspalum</i> <sup>a</sup> , <i>Tribolium</i> , <i>Triticum</i> , unidentified
Herb	197	92.9	<i>Chenopodium</i> , <i>Cyclosporum</i> , <i>Eclipta</i> , <i>Eleutheranthera</i> , <i>Erigeron</i> , <i>Euphorbia</i> , <i>Fallopia</i> , <i>Hyptis</i> , <i>Hypochaeris</i> , <i>Lepidium</i> , <i>Ludwigia</i> <sup>a</sup> , <i>Mimosa</i> , <i>Monopsis</i> , <i>Oldenlandia</i> , <i>Phyllanthus</i> , <i>Plantago</i> , <i>Polygonum</i> , <i>Portulaca</i> , <i>Sonchus</i> <sup>a</sup> , <i>Symphotrichum</i> , unidentified
Rush	13	100	<i>Juncus</i>
Sedge	60	96.7	<i>Cyperus</i> , <i>Fimbristylis</i> , unidentified
Trees	2	100	<i>Casuarina</i>
Unidentified	112	90.0	
Vine	1	100	<i>Macropitilium</i>

<sup>a</sup> Contains plant taxa regulated by New Zealand Ministry for Primary Industry (01 June 2023).

Nematode counts were significantly higher in samples from used machinery and shipping containers, than flat rack containers ( $p < 0.001$ ,  $Z = 4.9$  and  $4.0$ , respectively), with no significant difference between shipping containers and used machinery (Table 4). Counts of PPN from used machinery were significantly higher than from both shipping containers and flat rack containers ( $p = 0.004$ ,  $Z = 2.91$ , and  $p = 0.003$ ,  $Z = 2.94$ , respectively). There was no difference in seed counts between freight types (Table 4).

Nematodes were significantly more prevalent in soil from used machinery than shipping containers ( $p = 0.018$ ,  $Z = 2.36$ ) and flat rack containers ( $p = 0.028$ ,  $Z = 2.20$ ). PPN were not significantly different. Seed prevalence was also significantly higher in samples from used machinery than from shipping containers ( $p = 0.029$ ,  $Z = 2.18$ ) (Table 4).

### Culturable microorganisms

For bacteria, sample age had the greatest effect on bacteria counts ( $p = 0.001$ , Chi-square = 16.17,  $df = 1$ ), with no additional effects of sample weight being found after the sample age effect was accounted for (Table 5). The predicted response was that mean bacterial counts declined by 25% per 1 day increase in sample age.

For fungi, weight class had the most dominant significant effect on counts ( $p = 0.001$ , Chi-square = 25.47,  $df = 4$ ) (Table 5), with samples in three middle weight classes (21–100, 101–300 and 301–600 g) having larger fungal counts ( $1.62 \pm 0.37$ ,  $1.61 \pm 0.36$  and  $2.80 \pm 0.91 \times 10^5$ , respectively) compared to samples in the other two classes: 0–20 g and 601–5000 g ( $0.63 \pm 0.23$  and  $0.34 \pm 0.10 \times 10^5$ , respectively). After weight class effects were accounted for, no other factor/variable had a significant effect on fungi counts.

For *Pseudomonas*, sample departure season has the most significant effect on counts ( $p < 0.001$ , Chi-square = 21.38,  $df = 2$ ), with samples in autumn having smaller mean ( $\pm$ SEM) counts ( $0.38 \pm 0.08 \times 10^5$ ) than samples in spring and winter ( $4.40 \pm 3.31$  and  $1.26 \pm 0.36 \times 10^5$ , respectively). After accounting for sample departure season effects, no other factor/variable was significant.

**Table 4.** Mean number ( $\pm$  SEM) and % prevalence ( $\pm$ SE %<sup>1</sup>) of bacteria, fungi, *Pseudomonas* (CFU g<sup>-1</sup>), total and plant parasitic (PPN) nematodes, and plant seeds (g<sup>-1</sup> of soil contaminant) in soil removed from different types of sea freight arriving at Auckland and Lyttelton sea ports. *P* and Chi square values were derived from either negative binomial GLM analysis or zero-inflated count model depending on the characteristics of data being analysed.

Organism taxa	Shipping container (168)	Flat rack (9)	Used machinery (14)	Significance
Bacteria	1.95 × 10 <sup>7</sup> ± 2.13 × 10 <sup>6</sup> CFU <sup>a</sup>	1.19 × 10 <sup>7</sup> ± 5.42 × 10 <sup>6</sup> CFU <sup>a</sup>	2.74 × 10 <sup>7</sup> ± 1.02 × 10 <sup>7</sup> CFU <sup>a</sup>	NS
	99.4%	100%	100%	NS
Fungi	1.39 × 10 <sup>5</sup> ± 1.84 × 10 <sup>4</sup> CFU <sup>a</sup>	1.50 × 10 <sup>4</sup> ± 9.09 × 10 <sup>3</sup> CFU <sup>b</sup>	3.40 × 10 <sup>5</sup> ± 1.62 × 10 <sup>5</sup> CFU <sup>a</sup>	<i>p</i> < 0.001
	98.8%	100%	100%	
<i>Pseudomonas</i> spp.	5.35 × 10 <sup>4</sup> ± 9.47 × 10 <sup>3</sup> CFU <sup>b</sup>	1.31 × 10 <sup>3</sup> ± 1.02 × 10 <sup>3</sup> CFU <sup>c</sup>	7.84 × 10 <sup>5</sup> ± 4.89 × 10 <sup>5</sup> CFU <sup>a</sup>	<i>p</i> < 0.001
	82.0%	77.8%	85.7%	
Total nematodes	0.48 ± 0.15 <sup>a</sup>	0.001 ± 0.001 <sup>b</sup>	6.96 ± 6.04 <sup>a</sup>	<i>p</i> < 0.001
	37 ± 3.7% <sup>b</sup>	22 ± 14.6% <sup>b</sup>	71 ± 12.6% <sup>a</sup>	<i>p</i> < 0.028
PPN	0.02 ± 0.01 <sup>b</sup>	0.001 ± 0.001 <sup>b</sup>	0.65 ± 0.49 <sup>a</sup>	<i>p</i> < 0.001
	18 ± 3.0% <sup>a</sup>	16 ± 12.8% <sup>a</sup>	40 ± 13.6% <sup>a</sup>	NS
Seeds	0.09 ± 0.02 <sup>a</sup>	0.03 ± 0.03 <sup>a</sup>	0.09 ± 0.06 <sup>a</sup>	NS
	25 ± 3.3% <sup>b</sup>	36 ± 17.0% <sup>a,b</sup>	54 ± 13.8% <sup>a</sup>	<i>p</i> < 0.029

<sup>1</sup> The SE around the percentage mean *p* is calculated using the equation = 100 \*  $\sqrt{\frac{p(1-p)/100}{n-1}}$ , where n refers to number of samples. Values with the same letters not significantly different (*p* > 0.05).

## Nematodes and seeds

The most dominant effect for nematode counts was weight class (*p* < 0.001, Chi-square = 33.0, df = 4) with samples in the 21–100g class having larger counts (2.73 ± 1.65 g<sup>-1</sup> soil) than samples in other classes (e.g., 0–20g (0.24 ± 0.18), 101–300g (0.28 ± 0.10), 301–600g (0.22 ± 0.12) and 601–5000g (0.18 ± 0.13), respectively).

For PPN, log<sub>e</sub>-transformed weight covariate had the greatest effect on counts (*p* = 0.012, Chi-square = 8.8, df = 1), with the mean number of PPN decreasing by 41% for every 1 unit increase in log<sub>e</sub> weight. The predicted response for mean PPN counts was that if 152.7 g soil (overall median soil weight) contained 100 PPN g<sup>-1</sup> soil, 415.0 g soil would contain 59 PPN g<sup>-1</sup> soil.

For prevalence of total nematodes, UNRegion had the most significant effect (*p* = 0.002, Chi-square = 14.80, df = 3, binomial GLM) compared to the other significant variables with the highest prevalence in soil originating from Europe (100%, n = 3), followed by Oceania (39 ± 3.7%, n = 161), whereas there were no nematodes in soil from Asia and the Americas (0%, n = 9). For PPN prevalence, no variable was found to be significant.

For seed counts and seed prevalence, no factors/variables had any significant effects (Table 5).

## Position of soil on shipping containers

Overall, there were no significant differences in bacterial, fungal and *Pseudomonas* counts in soil collected from sea container roofs, outer rails, undersides and fix points (Table 6). However, nematode counts were absent in soil from roofs, with this absence

**Table 5.** Significance levels ( $p$ -values) of the effects of sample weight (class factor or  $\log_e$ -transformed covariate), sample origin (country, EcoRegion or UNRegion), sample age and sample departure season on counts of bacteria, fungi, *Pseudomonas*, and counts and % prevalence of total and plant parasitic (PPN) nematodes, and seeds, associated with soil removed from sea freight arriving at Auckland and Lyttelton sea ports. The most significant influencing variable indicated in bold.

Organism taxa	Sample weight		Sample origin			Sample age	Departure season
	Class factor	Covariate	Country	EcoRegion	UNRegion		
Bacterial count	< 0.001	< 0.001	0.793	0.203	0.219	< 0.001	0.287
Fungal count	< 0.001	0.862	0.040	0.045	0.195	0.118	0.235
<i>Pseudomonas</i> spp. count	0.003	0.689	< 0.001	0.007	0.013	0.014	< 0.001
Total nematodes count	< 0.001	< 0.001	0.019	< 0.001	0.014	0.201	0.002
Prevalence	0.221	0.022	0.101	0.009	<b>0.002</b>	0.064	0.591
PPN count	0.017	<b>0.012</b>	0.190	0.019	0.407	0.183	0.406
Prevalence	0.384	0.103	0.190	0.054	0.324	0.115	0.944
Seed count	0.647	0.150	0.429	0.221	0.111	0.527	0.165
Prevalence	0.450	0.115	0.940	0.760	0.842	0.737	0.774

**Table 6.** Mean number ( $\pm$  SEM) and % prevalence ( $\pm$ SE %<sup>1</sup>) of bacteria, fungi, *Pseudomonas* (CFU/g soil), total and plant parasitic (PPN) nematodes, and seeds (/g soil), by position on shipping containers. Comparisons were made in either negative binomial GLM analysis or zero-inflated count model analysis for numbers, depending on the characteristics of data being analysed. For prevalence, comparisons were by binomial GLM analysis. Prevalence of bacteria, fungi and *Pseudomonas* spp. were generally very similar, therefore not statistically compared.

Organism taxa	Roof (7)	Outer rail (36)	Underside (104)	Fix points (20)
Bacteria	$1.3 \times 10^7 \pm 8.44 \times 10^6$ CFU <sup>a</sup> 100%	$1.7 \times 10^7 \pm 4.22 \times 10^6$ CFU <sup>a</sup> 97.2%	$2.2 \times 10^7 \pm 3.13 \times 10^6$ CFU <sup>a</sup> 100%	$1.8 \times 10^7 \pm 5.87 \times 10^6$ CFU <sup>a</sup> 100%
Fungi	$1.1 \times 10^5 \pm 8.12 \times 10^4$ CFU <sup>a</sup> 85.7%	$1.2 \times 10^5 \pm 3.62 \times 10^4$ CFU <sup>a</sup> 100%	$1.7 \times 10^5 \pm 2.79 \times 10^4$ CFU <sup>a</sup> 99.0%	$7.9 \times 10^4 \pm 3.13 \times 10^4$ CFU <sup>a</sup> 100%
<i>Pseudomonas</i>	$2.9 \times 10^4 \pm 2.88 \times 10^4$ CFU <sup>a</sup> 85.7%	$3.7 \times 10^4 \pm 1.69 \times 10^4$ CFU <sup>a</sup> 83.3%	$6.7 \times 10^4 \pm 1.55 \times 10^4$ CFU <sup>a</sup> 84.6%	$3.9 \times 10^4 \pm 1.99 \times 10^4$ CFU <sup>a</sup> 73.7%
Total nematodes	0.0 <sup>c</sup>	$0.5 \pm 0.26^{ab}$	$0.2 \pm 0.06^b$	$1.5 \pm 0.86^c$
PPN	0.0% <sup>b</sup>	$32 \pm 8.6\%^a$	$39 \pm 4.9\%^a$	$42 \pm 11.2\%^a$
Seeds	0.0% <sup>a</sup>	$19 \pm 7.6\%^a$	$25 \pm 5.2\%^a$	$22 \pm 9.7\%^a$
	$0.01 \pm 0.01^a$ $38 \pm 21.9\%^a$	$0.03 \pm 0.01^a$ $29 \pm 8.2\%^a$	$0.03 \pm 0.01^a$ $22 \pm 4.1\%^a$	$0.03 \pm 0.02^a$ $34 \pm 10.7\%^a$

<sup>1</sup> The SE around the percentage mean  $p$  calculated using the equation =  $100 \times \sqrt{\frac{p \cdot (1-p)}{n-1}}$ . Letter n in the equation refers to number of samples. Values with the same letters are not significantly different ( $p > 0.05$ ).

being significant against other positions, while the counts from fix points ( $1.5 \pm 0.86$  g<sup>-1</sup> soil) were significantly higher than those from sea container undersides ( $p < 0.001$ , Chi-square = 18.8, df = 2) (Table 6).

Although numbers of PPN g<sup>-1</sup> soil were low ( $< 0.13$  g<sup>-1</sup>soil), significant differences in counts were found between positions, with PPN counts significantly higher ( $p < 0.001$ , Chi-square = 16.6, df = 2) in soil from outer rails, undersides and fix points compared to roof soil, where no nematodes were recovered. Significant differences were also found between the underside and outer rails ( $p = 0.012$ ,  $Z = 2.53$ ). The number of seeds g<sup>-1</sup> of soil did not significantly differ between the four positions (Table 6).

Total nematode prevalence was highest in container fix points ( $42 \pm 11.2\%$ ), followed by undersides ( $39 \pm 4.9\%$ ), and outer rails ( $32 \pm 8.6\%$ ), with no nematodes found in the roof soil. The absence in the roof soil was significant ( $p < 0.001$ , Chi-square = 22.5,  $df = 2$ ) against the other positions. The prevalence of PPN was relatively low, with no significant differences amongst shipping container positions. Seed prevalence did not significantly differ between the four positions, either (Table 6).

### Shipping containers alone

The sea container analysis showed similar relationships to the full dataset and detailed in Suppl. material 3.

## Discussion

Trade and commerce have been identified as key elements contributing to the spread of exotic species on a global scale. Not only does the spread of exotic species through these networks represent significant environmental, economic and social costs to natural and agricultural environments if IAS (a subset of exotic species) were to establish, a loss of biodiversity is also an expected consequence of IAS establishment. For islands, the implications can be significant, as they have high levels of endemism and IAS establishment can lead to extinction of species as well as biodiversity declines (Pyšek et al. 2020; Dawson et al. 2022).

The global movement of sea freight has an important role in the spread of IAS. In this study, it was found that soil on sea freight supported viable taxa either found in the soil (e.g., microorganisms, nematodes) or associated with the soil (e.g., arthropods). Soil was present on most types of sea freight, irrespective of origin, with all soil likely to vector microbes including plant pathogenic ones. About a third of soil contaminants contained live nematodes, some of which are plant feeders. Some of the taxa recovered are regulated organisms or found to be new interception records (e.g., *Pseudomonas*, nematodes, plants) under New Zealand biosecurity legislation. Importantly, this reinforces the importance of phytosanitary measures either pre- or at the border, to remove risk organisms and minimise the flow and potential for establishment of IAS. Seventy-three percent of soil was collected from the undersides of containers and flat racks, which was consistent with a previous shipping container survey that found most soil on container undersides (Gadgil et al. 2000). Similarly, 70% of soil in our study was obtained from shipping containers arriving from Pacific Islands. Gadgil et al. (2000) also found high rates of soil contamination on Pacific Island shipping containers. Although we also inspected containers from other regions (e.g., Asia and North America), superior hygiene standards in those regions meant the incidence of soil contamination was very low (and so not sampled). Flat rack containers had the highest soil contamination compared to shipping containers and used machinery, possibly because their design and role in carrying a wide range of freight in an open environment meant

a higher probability of being contaminated. However, despite a higher soil loading, it was found that the prevalence of fungi, *Pseudomonas* spp., nematodes and seeds were significantly lower than the other freight types. Counts of fungi, *Pseudomonas* spp., and nematodes were also significantly lower in flat rack containers compared to the two other freight types.

In terms of the variables analysed, the result was found to vary with taxa. Sample age was shown to have the most impact on survival of bacteria, while sample weight was the most important parameter for fungal counts, with a bell curve distribution whereby counts were significantly lower at both 0–20 g and >600 g weight classes, compared to weights >20–600 g. Why this trend occurred is not clear but may be an artefact of soil type or environmental exposure, or the interaction of both variables. For *Pseudomonas* counts, samples collected in spring and winter were significantly higher than autumn. For nematode counts, weight class was significant, while nematode prevalence was influenced by the region from which the soil originated, highest in soil from Europe. However, the small sample size from Europe ( $n = 3$ ) meant that the result should be treated with caution. For PPN,  $\log_e$ -transformed weight covariate had the greatest effect on counts.

When compared with the footwear study (McNeill et al. 2011), there were marked differences in the weight of soil collected and number and incidence of taxa. Perhaps not surprisingly, mean sample weight from sea freight was 417.3 g compared to 3.2 g for footwear (Table 7). While counts of culturable bacteria and fungi were comparable with those found on contaminated footwear, the incidence of nematodes, seeds and arthropods was higher in soil taken from footwear. Similarly, the number of nematodes and seeds per gram of soil was 45 × and 83 × higher in soil from footwear in comparison to sea freight (Table 7). Soil weight was the significant variable for fungi counts for both sea freight and shipping containers. However, in the footwear study, season was the most significant variable with higher fungi counts for samples collected in summer than in autumn.

A previous, controlled experiment that examined storage time and environmental exposure on survival of soil biota associated with shipping containers (McNeill et al. 2017), concluded that in general, the survival of soil biota is reduced by both duration of transport and exposure to sunlight, temperature and moisture fluctuations. Greater exposure of soil to abiotic parameters may explain why flat racks, despite supporting greater volumes of soil, had significantly lower counts and incidence of live taxa compared to shipping containers and used machinery. However, counts of fungi and bacteria can also be high from soil in exposed situations, probably due to ongoing colonisation by airborne propagules (McNeill et al. 2017). The sea freight results for nematodes were generally consistent with those of McNeill et al. (2017), because the percentage of soil found with nematodes was highest in the forklift points, the underside and outer rail, whereas prevalence was zero in roof soil. The prevalence of PPN was highest in soil collected from the underside of containers with no significant differences amongst the remaining shipping container positions. However, unlike McNeill et al. (2017) - but as in the footwear study (McNeill et al. 2011) - bacteria counts significantly declined with increasing soil age. Possibly the increase in fungal counts with time observed by

**Table 7.** Summary of organisms found associated with soil on sea freight in comparison with previous results from footwear.

	Sea freight	Footwear <sup>a</sup>
<b>Number of samples</b>	191	155
Sample weight (g)		
Mean per sample	417.3	3.2
Median per sample	152.7	1.0
<b>Bacteria</b>		
Bacteria incidence	0.99	1.00
Bacteria (CFU/ gram of soil)	$2.1 \times 10^7$	$2.9 \times 10^7$
<b>Fungi</b>		
Fungi incidence	0.99	0.98
Fungi (CFU/ gram of soil)	$1.4 \times 10^5$	$5.6 \times 10^5$
<b>Nematodes</b>		
Nematode incidence	0.38	0.65
Mean nematodes/ gram of soil	0.9	41
<b>Seeds</b>		
Seed incidence	0.28	0.52
Mean seeds/ gram of soil	0.03	2.5
Proportion grass	0.67	0.44
Proportion herb	0.23	0.13
Proportion trees and shrubs	0.02	0.39
<b>Arthropods</b>		
Incidence of arthropods or arthropod-parts	0.20	0.38
Proportion of arthropods alive	0.18	0.03

<sup>a</sup> McNeill MR, Phillips CB, Young S, Shah F, Aalders L, Bell N, Gerard E, Littlejohn R (2011) Transportation of nonindigenous species via soil on international aircraft passengers' footwear. *Biological Invasions* 13: 2799–2815. <https://doi.org/10.1007/s10530-011-9964-3>.

McNeill et al. (2017) occurred because their experiment was conducted entirely on land where airborne fungal spores may be more abundant than at sea where the sea freight samples in the present study spent some of their time. This study found no significant differences in counts or prevalence of seeds, bacteria, fungi and *Pseudomonas* spp. amongst samples sampled from four different locations on sea containers.

Nineteen different nematode families or species were found in sea freight soil, which was comparable to the results of the footwear study (McNeill et al. 2011), where six trophic groups containing 17 families or species were found. The types of nematodes detected were also similar with Rhabditidae, *Panagrolaimus*, Plectidae, *Aphelenchoides*, *Aphelenchus*, *Ditylenchus* and Omnivores being common to both studies. As noted previously, nematode counts in soil from sea freight were significantly higher in soil between 21–100g, compared to weights below and above the range. By comparison, in footwear there was a significant increase with increasing soil weight (McNeill et al. 2011).

Various species of PPN were identified from the samples, some of which are regulated pests in New Zealand, thus showing that sea freight can act as a pathway for the movement for regulated species. *Aphelenchoides besseyi* (regulated) causes white tip disease in rice and 'summer crimp' in strawberries, and also feeds on other plants and fungi (Hunt 1993). *Aphelenchoides fujianensis* (regulated) occurred in soil on a shipping container from Vanuatu; little is known about this species, but it is probably

mycophagous (Zhuo et al. 2010). Another shipping container specimen from Vanuatu was probably *A. bicaudatus* (regulated), which feeds on cultivated mushrooms (Hunt 1993). *Aphelenchus avenae* has been previously recorded in New Zealand and is associated with mushrooms (Knight et al. 1997). *Neopsilenchus magnidens* (Thorne) Thorne & Malek, was collected off used machinery from Italy and is associated with alfalfa in the USA (Siddiqi 2000). *Helicotylenchus dihystra* (Cobb) Sher, found in soil from Tonga, and *Ditylenchus dipsaci* (Kühn) Filipjev (stem nematode), recovered from both a shipping container from Tonga and from a stump grinding excavator from Australia are already present in New Zealand (Knight et al. 1997).

*Pseudomonas* isolates, representing the most dominant colony types on PSA for each sea freight soil sample, covered the three *Pseudomonas* lineages and nine of the fourteen phylogroups (Mulet et al. 2010; Passarelli-Araujo et al. 2022; Peix et al. 2018). *Pseudomonas stutzeri* was the most dominant phylogroup (51%) followed by the *P. putida* phylogroup, (25%) (Suppl. materials 1, 2: fig. S2, table S4). Isolates were not identified to species level due to the complexity of *Pseudomonas* taxonomy with species identification requiring the sequencing of at least three house-keeping genes in addition to the 16S rRNA gene (Mulet et al. 2010). Furthermore, testament to the rapidly evolving field of *Pseudomonas* taxonomy is the proposal of several new genera within the Pseudomonadaceae family which include species formerly ascribed to the *Pseudomonas* genus (Hesse et al. 2018; Gomila et al. 2022; Lalucat et al. 2022). A general understanding of the genetic diversity within the *Pseudomonas* isolates was therefore appropriate for this study.

Twenty-five plant-pathogenic *Pseudomonas* spp. have been described (Höfte and De Vos 2007; Bull et al. 2010, 2012), with *P. syringae* van Hall, considered a bacterial plant pathogen with significant impact (Mansfield et al. 2012). Godfrey and Marshall (2002) previously showed that exotic soil attached to shipping containers was a potential source of new pseudomonad biodiversity into New Zealand. Neither *P. stutzeri* nor *P. putida* are known as plant pathogens (Bull et al. 2010, 2012). *Pseudomonas stutzeri* occupies diverse ecological niches and has been isolated as an opportunistic pathogen from humans (Lalucat et al. 2006). *Pseudomonas putida* is a metabolically versatile saprophytic soil bacterium and appears to lack genes for the proteins and enzymes that cause disease in plants (Nelson et al. 2002). Five isolates (AUS50i, AUS139b, AUS140a, ITA154c and UNK162a), that originated from Australia, Italy and the United Kingdom, grouped with the *P. fluorescens* phylogroup which is closest to the *P. syringae* phylogroup where the highly virulent plant pathogenic *P. syringae* pathovars reside (Suppl. material 1: fig. S2). The results of the sequence analysis demonstrated the high species diversity in the *Pseudomonas* sea freight isolates and the potential for soil on sea freight to transport high risk species.

The diversity of *Pseudomonas* spp. found in this study also aligns well with Godfrey and Marshall (2002), who found a wide distribution of soil isolates throughout the *Pseudomonas* genus from imported shipping containers. In contrast, an earlier study on shipping containers arriving at New Zealand ports showed much lower *Pseudomonas* spp. diversity (Marshall and Varney 2000). In both the Marshall and Varney (2000) and Godfrey and Marshall (2002) studies, *P. putida* was the dominant species.

Twenty eight percent of samples contained seeds including the genera *Brachiaria*, *Cortaderia*, *Digitaria*, *Eragrostis* (grasses) and *Sonchus* (herb), which include biosecurity risk species regulated in New Zealand. Grass and herb genera (n = 16 and 21, respectively) were the dominant plant types, which was a similar diversity to the footwear study (McNeill et al. 2011). However, there was little commonality in the genera found with only three grass (*Eragrostis*, *Paspalum*, *Triticum*) and one herb (*Polygonum*) genus occurring in both studies. This dissimilarity may have arisen from geographic differences in the sample sources: sea freight was mainly from the Pacific Islands and Australia, whereas footwear was mainly from the UK, Australia, Central Europe and North America. The difference may also relate to the mobility of humans and the potential for footwear to be contaminated from more diverse environments, compared to that of sea freight which is mainly associated with road and rail transport networks.

Only 20% of the samples contained live and recognizable dead arthropods, but they included taxa that are known to, or have potential to, impact native fauna (e.g., spiders, Eulophid wasps), are invasive (e.g., ants) and potential impacts on animal health (e.g., flies). The wasp nest containing the cadaver of eumenid wasp *Delta esuriens* was of interest. The wasp is introduced to the Pacific, and while reported in the Cook Islands and Samoa, was not known from Tonga from which the container originated. *Delta esuriens* has not been reported in New Zealand.

Whether these results allow for development of recommendations for prioritising one pathway over another, to increase the efficiency and efficacy of biosecurity interventions is open to debate. Comparison of sea freight compared to footwear indicate that both pathways vector biosecurity risk organisms, although the level of risk did vary, with the incidence, counts and diversity in soil on external surfaces of shipping containers less than soil transported in more protected environments (e.g., on footwear in luggage). Prioritising effort must also be viewed in relation to other pathways along which exotic species can move (Hulme et al. 2008). The conservative approach would deem both pathways important and therefore require biosecurity intervention. This is particularly so because interventions (e.g., cleaning), address a range of taxa collectively posing a potential biosecurity risk. In addition, pathways for biological invasions are dynamic and priorities may shift depending on new information, such as new pests or diseases (Barnes et al. 2018), or secondary invasions that provides new pathways for exotic species to disperse (Bertelsmeier and Keller 2018).

Over 2011 and 2012, the years in which the study was undertaken, approximately 456,680 empty and 750,500 full containers arrived at New Zealand seaports (MPI, unpublished data). Of these, the majority of sea containers were from Australia (c. 33%), followed by China (13%), Singapore (9.4%) and Malaysia (7%). The Pacific Islands were the origin for 6.3% of the sea container freight. Over the same time frame, c. 20,000 used vehicles were imported into the country. External contamination of shipping containers has been found to vary from 1 (M. R. McNeill, unpublished data) to 10% (Anon, 2016), and 1–50% for used vehicles (Hustedt 2010). This study therefore sampled a fraction of the shipping containers and used machinery arriving in New Zealand each year that carried some form of contamina-

tion. Nevertheless, we were able to detect numerous diverse exotic organisms, some of which were regulated pests.

In 2011, the volume of TEUs (20-foot equivalent units) moved globally by maritime shipping was 153 million, but grew by 4.6% in 2013, taking total volumes to 160 million TEUs (UNCTAD 2014, page 17). By 2018, containerised trade reached 196 million TEUs (Clarksons Research 2019), and while the Covid-19 pandemic and trade tensions have impacted maritime trade (UNCTAD 2020), Clarksons Research predicted trade volumes would reach 206.8 million TEUs in 2021 (Chambers 2021). Even if only 1% of shipping containers and sea freight were contaminated with soil, our results suggest they must facilitate significant exchange of exotic species across and within countries. Our results therefore support Dawson et al. (2022), who found that shipping containers were the primary introduction pathway for IAS to United Kingdom Overseas Territories. Managing the risk posed by sea freight is critical if a country's environment, biodiversity and economy is to be protected.

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## Supplementary material I

### Sampling points on sea container and visualisation of the genetic diversity between the 16S rRNA genes

Authors: Mark R. McNeill

Data type: pdf

Explanation note: **fig. S1.** Sampling points on sea container indicating roof, outer rail, underside (inner rails or bottom of container), fork lift pockets and corner castings. Examples of contamination recorded during the study are shown. **fig. S2.** Visualisation of the genetic diversity between the 16S rRNA genes of 77 sea freight soil isolates and 21 *Pseudomonas* reference strains. The isolates from this study are named using the GenBank accession numbers (KX670872-KX670948) followed by a three-letter acronym denoting the country that the shipping container originated from: AUS Australia, CHR Christmas Island, FIJ Fiji, ITA Italy, MAL Malaysia, SAM American Samoa, TAI Thailand, TGA Tonga, UNK United Kingdom, USA United States of America, VAN Vanuatu and WFI Wallis & Futuna Islands. Phylogroups fall in the following *Pseudomonas* lineages: *P. aeruginosa* (*P. acaligenes*, *P. aeruginosa*, *P. oryzihabitans*, *P. oleovorans*, *P. stutzeri*), *P. fluorescens* (*P. anguilliseptica*, *P. fluorescens*, *P. putida*, *P. syringae*) and *P. pertucinogena* (*P. pertucinogena*). Using MEGA11, distance was calculated by the Jukes-Cantor model and visualised using a neighbour-joining tree. The robustness of the nodes was assessed with 1000 bootstrap replicates and values showing  $\geq 50\%$  support are shown.

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Link: <https://doi.org/10.3897/neobiota.88.98440.suppl1>

## Supplementary material 2

### Soil contamination data, Insect taxa, *Pseudomonas* isolates and reference species, and plant taxa identified by molecular analysis

Authors: Mark R. McNeill

Data type: pdf

Explanation note: **table S1.** Details on amount of soil (g) collected from shipping containers, flat rack containers and used machinery at Auckland and Christchurch sea ports per unit (individual freight or machinery item) and per sample. **table S2.** Number and weight of soil removed from sea container and flat rack containers sampled at Auckland and Lyttelton sea ports. **table S3.** Live insect taxa recovered directly from soil removed from shipping containers arriving at Auckland and Lyttelton sea ports. **table S4.** *Pseudomonas* isolates from soil removed from sea freight arriving into Auckland and Lyttelton sea ports. Assigned to previously described *Pseudomonas* phylogroups and lineages (Mulet et al. 2010; Peix et al. 2018; Passarelli-Araujo et al. 2022). **table S5.** Twenty-one reference species of the genus *Pseudomonas* used in the 16S rRNA gene nucleotide-based distance analysis. Isolated from soil recovered from sea containers arriving at Auckland and Lyttelton sea ports. **table S6.** Plant species identified by molecular analysis from soil collected from sea freight arriving at Auckland and Christchurch sea ports.

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## Supplementary material 3

### Analysis of shipping container data only

Authors: Mark R. McNeill

Data type: docx

Explanation note: Effects of soil weight, origin, source, sample age and season bacteria, fungi and *Pseudomonas*.

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# Invasive alien plants in South Asia: Impacts and management

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## Abstract

South Asia is home to an immense diversity of flora and fauna, which makes it one of the global biodiversity hotspots. Plant invasions are one of several factors that threaten South-Asian biodiversity. This review lists problematic invasive plant species, analyses their negative impacts, and summarises management methods implemented in South Asia using data obtained from research articles and relevant databases (CABI, GISD, GloNAF). The data was used to evaluate the research trends over time, knowledge of the impacts of invasive plants, and management measures aimed at the invasive species. In total, 392 currently invasive vascular plant species were recorded in South Asia. Of these, 41 species are widely distributed in South Asia, occurring in at least three countries, and 20 species that are listed as invasive in South-Asian countries by the book *Invasive Plant Species of the World* are considered as the most problematic. For a subset of the most problematic species where such information is available, we present management measures that are in place in individual countries. The number of studies on invasive species in South Asia has been increasing, with more than half (53%) represented by local and regional inventories. Among the countries in South Asia, India has the highest number of invasive (145) and naturalized plant species (471). However, the percentage contribution of invasive and naturalized species to the native flora is the highest in the Maldives Islands. Studies on impacts are limited to those on native plants and agriculture; there is a lack of research on impacts on ecosystems and hydrology, as well as on economic costs and human health. Moreover, impacts have been quantified for very few species. Currently, the management of invasive plants is mostly done by physical or mechanical methods; research into opportunities for biological control is inadequate. Our review highlights the urgent need to quantify the impacts of all prevalent and problematic invasive species in South Asia as a crucial step in allocating resources for their management and addressing the knowledge gap in this region.

**Keywords**

Biodiversity, biological control, invasions, inventories, naturalized species

**Introduction**

A species that is introduced outside of its native range due to intentional and unintentional human activity is considered an alien species (Richardson et al. 2000; Pyšek et al. 2004). Alien species creating self-sustaining populations in the invaded region are termed naturalized species, and a subset of naturalized species that rapidly spread in the invaded region from the site of its original introduction are considered invasive (Richardson et al. 2000; Pyšek et al. 2004; Blackburn et al. 2011). Some definitions consider only those alien species as invasive that have negative impacts on the environment (IUCN 2000). Invasive species grow fast, become widespread, form self-sustaining populations, produce large numbers of reproductive offspring, and can grow in a range of habitats, such as agricultural land, grassland, wasteland and other ruderal habitats, dry land, and riparian habitats (Chytrý et al. 2008, 2009; Patzelt et al. 2022; Pyšek et al. 2022).

Biological invasions are considered the fifth most important driver of global environmental change (IPBES 2019). Of the global plant species pool, ~14,000 taxa are known to have naturalized, i.e. ~4% of the world flora (van Kleunen et al. 2015, 2019), and ~2500 species are considered invasive (Pagad et al. 2018), with the Asteraceae family contributing the highest number of naturalized taxa (Pyšek et al. 2017). The number of invasive species has increased globally due to escalating international trade (Seebens et al. 2015). The highest numbers of invasive plant species are reported from California (USA), Cuba, Florida (USA), India, Japan, South Africa, and Queensland (Australia) (Pyšek et al. 2017). Many countries have databases of invasive alien plants, but still, there is a lack of comprehensive information, which hampers efforts to develop and implement the policies for effective management (van Kleunen et al. 2015).

The impacts of invasive species on ecosystems and the environment are well documented in Europe (Kumschick et al. 2015; Nentwig et al. 2018; Langmaier and Lapin 2020) and North America (Duenas et al. 2018) in the Northern hemisphere, and South Africa (van Wilgen et al. 2020; McGaw et al. 2022; Richardson et al. 2022), New Zealand (Brandt et al. 2021) and Australia in the Southern Hemisphere. In addition to this, several databases such as GISD (Global Invasive Species Database; [www.iucngisd.org](http://www.iucngisd.org)), GRIIS (Global Register of Introduced and Invasive Alien Species; [www.griis.org](http://www.griis.org); Pagad et al. 2018), CABI (Invasive Species Compendium; <https://www.cabi.org/ISC>), GloNAF (Global Naturalized Alien Flora; van Kleunen et al. 2015, 2019; Pyšek et al. 2017), and DAISIE (Delivering Alien Species Inventories for Europe) (DAISIE 2009; Hulme et al. 2010) provide data for particular regions, which could

help with prioritization of problematic species in particular countries. However, there are geographical and taxonomical biases in invasion ecology (Pyšek et al. 2008, 2017).

South Asia includes eight countries: Afghanistan, Bangladesh, Bhutan, India, Pakistan, Maldives, Nepal, and Sri Lanka. It is surrounded by the Himalayas in the north and the Indian Ocean in the south. South Asia covers about 5.2 million km<sup>2</sup>, which is about 11.7% of the Asian continent and 3.5% of the world's land surface area. The climate varies, ranging from tropical monsoon in the south to a temperate climate in the north. South Asia overlaps with three biodiversity hotspots (Himalaya, Indo-Burma, and Western Ghats – Sri Lanka), harbouring 15.5% of global floral diversity (<http://www.sacep.org>). Invasive plants threaten Himalayan biodiversity, which is exceptionally rich in terms of diversity and endemism (Kumar and Scheiter 2019; Gupta et al. 2021). Climate change and anthropogenic pressure increase the problems caused by invasive species in these pristine regions (Mungi et al. 2018). With increasing trade, travel, and tourism, this trend is unlikely to stop in the near future (Early et al. 2016), so monitoring biodiversity-rich areas is important to identify the status of invasive species and implement proper management.

The socioeconomic problems caused by plant invasions are escalating on all continents. The direct cost from damage by invasive species is thirteen times higher than that incurred by management (Diagne et al. 2021). An analysis between 1970 and 2017 showed that the minimum estimated cost of biological invasion worldwide to human societies was US\$ 1.288 trillion (Diagne et al. 2021, but see Novoa et al. 2021). Economic costs due to biological invasions are comparably high in South Asia (US\$ 185.8 billion; Liu et al. 2021), and agriculture is the most affected sector. These costs have increased markedly in the past decades and do not show any sign of slowing down. In India alone, the estimated economic cost is US\$ 176.7 billion; for Bhutan, Maldives, Pakistan, and Sri Lanka, the cost is estimated to be less than US\$ 15 billion. However, no cost estimation has been done for Nepal, Bangladesh, and Afghanistan (Diagne et al. 2021).

Despite many individual studies, a comprehensive overview of plant invasions and their impacts and management has been missing from South Asia (Early et al. 2016; Shrestha et al. 2022). Due to high population density and ongoing environmental changes, including biological invasions, biodiversity in South Asia is under threat (IPBES 2019). Managing invasive species without baseline data and knowledge of their introduction pathways is difficult. Thus, region-wise or country-wise, detailed, up-to-date inventories of alien species are urgently needed. There is still a gap in the availability of data on alien species distribution in Asia, which is a constraint to synthesizing global data and trends and prevents the development of management strategies (Shrestha et al. 2022). Understanding the current state of plant invasions in South Asia will help to suggest new approaches for effective management.

To bridge the knowledge gaps in this region, we (i) analysed the temporal trends in topics associated with alien species research in South Asia and (ii) compiled a checklist containing the total number of naturalized and invasive species for the region. Further,

(iii) for the widespread and most problematic invasive plants, we collated information on their impacts, types of invaded habitats, control methods being used, and management implemented in South-Asian countries. The information presented in this paper can be used to improve the management of invasive plants and prioritize the most pressing research areas in this region.

## Methods

We searched research papers from Scopus, CABI, Web of Science, and Google Scholar, published from January 1977 to January 2022. The keywords used for the search were “invasive/alien/non-native/exotic, plant/flora/species” in the X where X is the name of a South-Asian country (Afghanistan, Bangladesh, Bhutan, India, Nepal, Maldives, Pakistan, and Sri Lanka). A total of 468 research papers were identified. Abstracts were scanned to select the relevant papers that were inspected in detail to determine whether they contained relevant data; 96 papers were excluded as being not peer-reviewed, reports, theses, conference proceedings, published in predatory journals, or otherwise irrelevant. The remaining 372 papers were used for the analysis (Supplementary Material 1). Based on the year of publication, the articles were used to evaluate the research trend over time and classified into seven research topics: allelopathy (chemical substances of invasive plants that affect other plants), climate change (its effects on the distribution of invasive species), species distribution (studies on spatial patterns of alien species), ecology (relationships between invasive species and its environment), impacts (evaluating the risk from invasion on native diversity and ecosystems), inventory (checklist and identification of species), and management (efforts made to limit the spread of invaders).

In addition to the literature review, databases such as GISD (Global Invasive Species Database; [www.iucngisd.org](http://www.iucngisd.org)), CABI Invasive Species Compendium; CABI 2022), GloNAF (Global Naturalized Alien Flora; van Kleunen et al. 2015, 2019; Pyšek et al. 2017) and GRIIS (Global Register of Introduced and Invasive Alien Species; [www.griis.org](http://www.griis.org); Pagad et al. 2018) were used to explore the status of invasive species in South-Asian countries. The numbers of naturalized and invasive species for individual countries were taken from GloNAF; for Bhutan, which was not included in GloNAF, we used other published information (Dorjee et al. 2020). As there is a large variation in the area of South-Asian countries, to make the numbers of species more comparable, we standardized the species number per log area.

The list of the most problematic invasive plants analysed here was based on the book “Invasive Plant Species of the World” (Weber 2017) and resulted from including all species that this book reports to occur as invasive in the South-Asian countries under study. Selecting the most problematic species with reference to one comprehensive source evaluating regional invasions by using comparable rigorous criteria (i.e. distribution and impact; Weber 2017) provides a balanced perspective of the current invasion load in South Asia and allows for assessing the threat from ongoing and future invasions in a broader view.

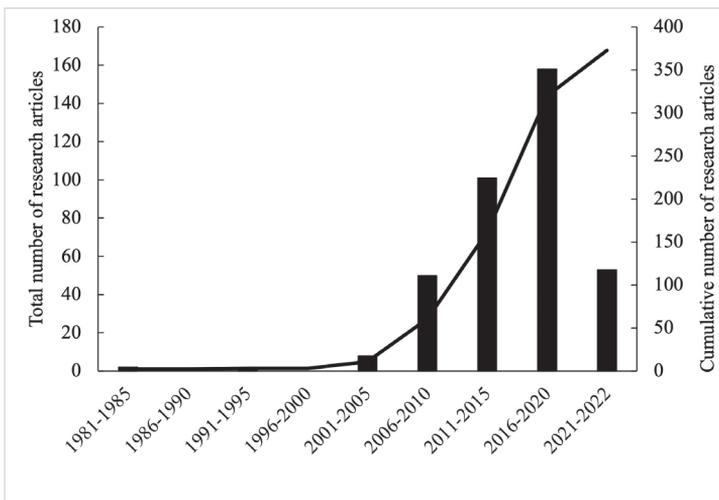
To determine the impacts of the most problematic invasive species, we used the following categories: impacts on plant diversity, soil, biodiversity, agriculture, socio-economy, health, hydrology, and livestock; the information on impacts was compiled from Weber (2017), CABI (2022) and Global Invasive Species Database (2022). For each species, we present, based on information available in the literature (Suppl. material 1), the overview of management measures that are used against them and in which countries.

We classified the most problematic invasive species according to the habitats in which they grow based on information from the same sources that were used to compile the list. We used the following habitat categories: Disturbed sites are abandoned sites or areas affected by anthropogenic activities, and riverine or riparian are the habitats in stream corridors. Grasslands include rangeland and pastures. Forest and forest edges represent closed canopy and open forest, respectively.

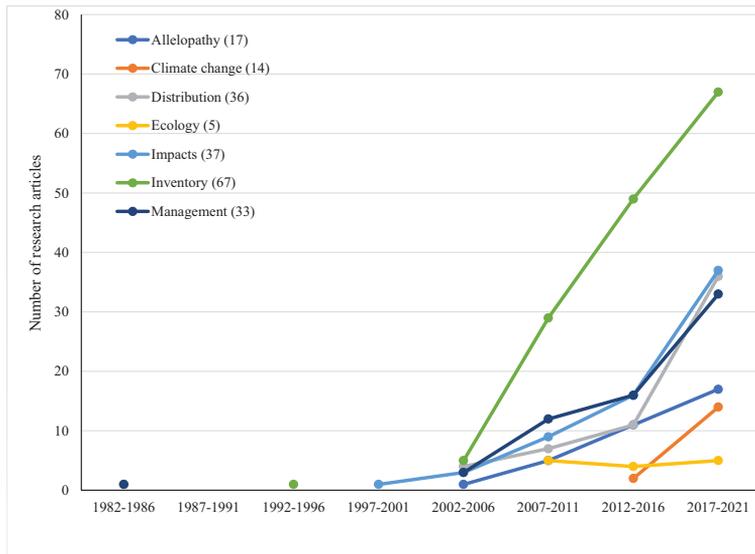
## Results

### Temporal trends in research on South Asia

Until 2000, studies on invasive alien plants were scarce in South Asia. Only after 2001 did the number of studies start to increase rapidly (Fig. 1), and this trend still holds for all categories of research. Most research (41% of studies) was focused on the inventories of invasive plants, followed by studies on impacts (18%) and distribution of alien species (16%) in South-Asian countries (Fig. 2). Among all countries in South Asia, India was the first to start research on alien trees and shrubs in 1983.



**Figure 1.** Numbers of research articles dealing with plant invasions in South-Asian countries in five-year periods and their cumulative number over the period of 1981–2022. See Suppl. material 1 for the articles on which the figure is based.



**Figure 2.** Number of articles addressing different research topics in South-Asian countries over time. See Suppl. material 1 for the articles on which the figure is based.

The majority of the studies addressing the consequences of plant invasions were focused on the impacts of invaders on native plant diversity (46% of the total number of articles dealing with impact), followed by studies on soils (17%), biodiversity on other trophic levels (15%), agriculture (14%), socioeconomic impacts (6%), and human health (3%). Except for soil studies, there is very little research regarding the impacts on ecosystems, including hydrology.

### South-Asian naturalized and invasive plants: the numbers

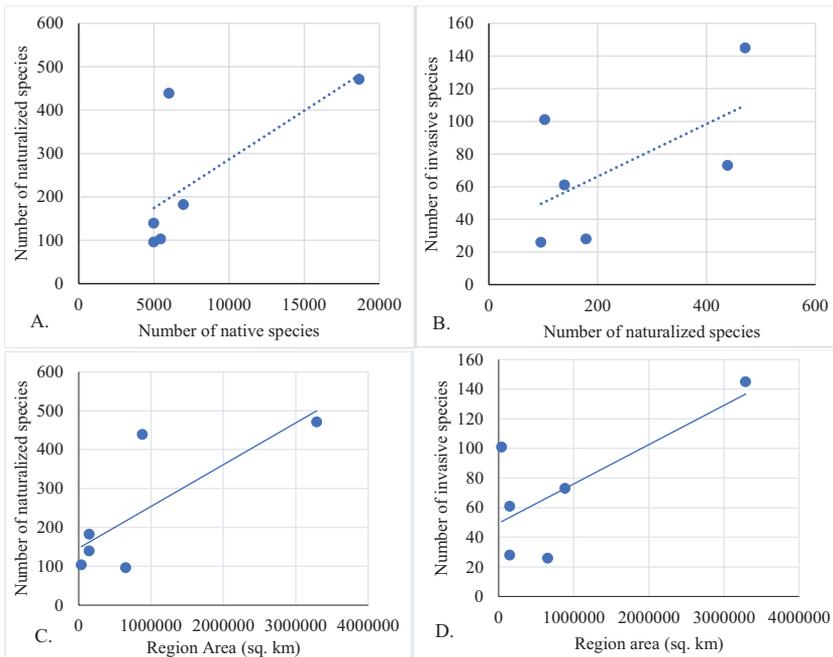
We recorded 392 alien plant species that are invasive in South Asia. India harboured the highest number of invasive plant species (145), followed by Bhutan (101), Sri Lanka (94), Pakistan (73), Bangladesh (61), Maldives (38), Nepal (28), and Afghanistan (26). The numbers of naturalized species followed a similar pattern, with India (471), Pakistan (439), and Sri Lanka (401) harbouring the most. The ranking of countries shifted if species numbers per log area were taken as a measure, with India appearing the richest in invasive and Sri Lanka, Pakistan, and India in naturalized species (Table 1). Although India, which is the largest country in the studied regions in terms of area, harboured the highest numbers of invasive and naturalized plant species, Maldives had the highest percentages of these species in its flora. Due to the rich flora of South Asia, the percentage of naturalized plant species across the whole region was rather low, only 3.9% of the total flora. Afghanistan is the third largest country (after India and Pakistan), but the number of naturalized and invasive species recorded there was the lowest; however, this may be due to a lack of research. The total numbers of naturalized, invasive, and native species reported from the reviewed countries are shown in Table 1.

**Table 1.** The number of invasive species, naturalized species, and percentage of naturalized species in the total flora of South-Asian countries as recorded in the GloNAF database (van Kleunen et al. 2019) and updated by other sources. Normalized invasive species value is obtained by dividing the number of invasive species ( $S_{inv}$ ) by the logarithm value of the country area and for the naturalized species as  $S_{nat}/\log Area$ .

Country	Invasive no. ( $S_{inv}$ )	Naturalized no. ( $S_{nat}$ )	Native no.	Naturalized %	Area	Invasive per log area	Naturalized per log area
Afghanistan	26	96	5,000	1.9	652,230	4.5	16.5
Bangladesh	61	139	5,000	2.8	147,570	12.2	26.9
Bhutan	101 <sup>c</sup>	204 <sup>c</sup>	5,446	1.9	38,394	22.0	22.5
India	145 <sup>a</sup>	471 <sup>b</sup>	18,664	2.5	3,287,590	54.0	72.3
Maldives	38	167	277	60.3	300	15.3	67.4
Nepal	28 <sup>d</sup>	182 <sup>d</sup>	6,973	2.9	147,181	5.2	35.2
Pakistan	73	439	6,000	7.3	881,912	12.3	73.8
Sri Lanka	94	401	3,368	11.9	65,610	20.1	83.3
<b>TOTAL</b>	<b>392</b>						

<sup>a</sup>Modified from Khuroo et al. 2021; <sup>b</sup>Modified from Inderjit et al. 2018; <sup>c</sup>Modified from Dorjee et al. 2020; <sup>d</sup>Modified from Shrestha et al. 2021 and Adhikari et al. 2022

The relationships between the number of naturalized and native species (Fig. 3A) and the number of invasive and naturalized species (Fig. 3B) were not significant. The numbers of naturalized and invasive species on the mainland significantly increased with the increasing area of the country (Fig. 3C, D).



**Figure 3.** Relationships of alien plant species numbers in the South-Asian region for mainland states **A** naturalized species relationship with native species (mainland:  $r=0.77$ ,  $t=0.59$ ,  $p=0.12$ ,  $df=5$ ) **B** invasive species relationship with naturalized species (mainland:  $r=0.67$ ,  $t=-0.11$ ,  $p=0.91$ ,  $df=5$ ) **C** species area relationship for naturalized species (mainland:  $r=0.77$ ,  $t=2.83$ ,  $p=0.04$ ,  $df=5$ ) **D** species area relationship for invasive species (mainland:  $r=0.92$ ,  $t=1.00$ ,  $p=0.008$ ,  $df=5$ ).

## The most widely distributed invasive plants in South-Asian countries

Forty-one invasive species occur in at least three South-Asian countries; we considered such species as widespread. *Lantana camara* and *Pontederia crassipes* are the most widely distributed, occurring in all eight South-Asian countries. *Parthenium hysterophorus* occurs in seven countries, *Chromolaena odorata* and *Mimosa pudica* in six countries, *Ageratum conyzoides*, *Argemone mexicana*, *Leucaena leucocephala*, *Mikania micrantha*, and *Ricinus communis* in five countries. Of the 41 widespread species, six are listed among 100 of the world's worst invasive species (see Table 2 for distribution of the most widespread invasive species in South-Asian countries).

**Table 2.** The distribution of widespread invasive plant species that were recorded in at least three of the eight studied countries. Based on GISD ([www.iucngisd.org](http://www.iucngisd.org)), CABI 2022, GloNAF (van Kleunen et al. 2019) and GRIIS (Pagad et al. 2018).

Species	Afghanistan	Bangladesh	Bhutan	India	Maldives	Nepal	Pakistan	Sri Lanka
1 <i>Lantana camara</i> L.*	✓	✓	✓	✓	✓	✓	✓	✓
2 <i>Pontederia crassipes</i> (Mart.) Solms*	✓	✓	✓	✓	✓	✓	✓	✓
3 <i>Parthenium hysterophorus</i> L.	✓	✓	✓	✓		✓	✓	✓
4 <i>Chromolaena odorata</i> (L.) R.M. King & H. Rob.*	✓	✓	✓	✓		✓		✓
5 <i>Mimosa pudica</i> L.	✓	✓	✓	✓		✓		✓
6 <i>Ageratum conyzoides</i> L.		✓	✓	✓		✓		✓
7 <i>Argemone mexicana</i> L.	✓	✓	✓	✓		✓		
8 <i>Leucaena leucocephala</i> (Lam.) de Wit*	✓	✓			✓		✓	✓
9 <i>Mikania micrantha</i> Kunth*		✓	✓	✓		✓		✓
10 <i>Ricinus communis</i> L.	✓		✓	✓	✓		✓	
11 <i>Acanthospermum hispidum</i> DC.		✓	✓	✓				✓
12 <i>Ageratina adenophora</i> (Spreng.) R. M. King & H. Rob.			✓	✓		✓		✓
13 <i>Alternanthera philoxeroides</i> (Mart.) Griseb.		✓		✓		✓		✓
14 <i>Amaranthus spinosus</i> L.			✓	✓		✓	✓	
15 <i>Ipomoea carnea</i> Jacq. subsp. <i>fistulosa</i> (Mart. ex Choisy) D. F. Austin		✓		✓		✓	✓	
16 <i>Mesosphaerum suaveolens</i> (L.) Kuntze		✓	✓	✓		✓		
17 <i>Pistia stratiotes</i> L.		✓				✓	✓	✓
18 <i>Prosopis juliflora</i> (Sw.) DC.				✓	✓		✓	✓
19 <i>Senna occidentalis</i> (L.) Link		✓		✓		✓	✓	
20 <i>Xanthium strumarium</i> L.			✓			✓	✓	✓
21 <i>Acacia auriculiformis</i> A. Cunn. ex Benth.	✓	✓						✓
22 <i>Acacia mangium</i> Willd.	✓	✓						✓
23 <i>Ageratum houstonianum</i> Mill.				✓		✓		✓
24 <i>Alternanthera pungens</i> Kunth				✓			✓	
25 <i>Amaranthus viridis</i> L.							✓	✓
26 <i>Bidens pilosa</i> L.			✓	✓		✓		
27 <i>Cannabis sativa</i> L.			✓	✓			✓	
28 <i>Casuarina equisetifolia</i> L.				✓	✓			✓

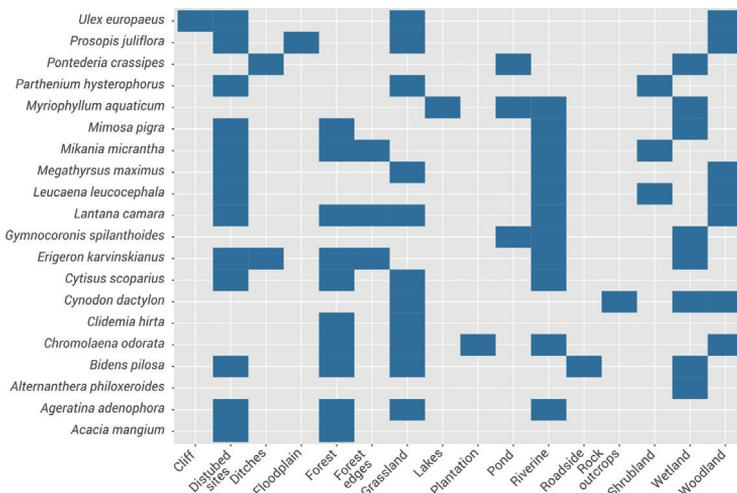
Species	Afghanistan	Bangladesh	Bhutan	India	Maldives	Nepal	Pakistan	Sri Lanka
29 <i>Croton bonplandianum</i> Baill.		✓	✓	✓				
30 <i>Datura stramonium</i> L.			✓	✓			✓	
31 <i>Erigeron karvinskianus</i> DC.				✓		✓		✓
32 <i>Eucalyptus camaldulensis</i> Dehnh.	✓	✓					✓	
33 <i>Galinsoga quadriradiata</i> Ruiz & Pav.			✓	✓		✓		
34 <i>Ipomoea quamoclit</i> L.			✓	✓	✓			
35 <i>Mikania scandens</i> (L.) Willd	✓	✓						✓
36 <i>Opuntia dillenii</i> Haw.		✓		✓				✓
37 <i>Oxalis latifolia</i> Kunth			✓	✓		✓		
38 <i>Portulaca oleracea</i> L.			✓	✓	✓			
39 <i>Robinia pseudoacacia</i> L.			✓	✓			✓	
40 <i>Senna alata</i> (L.) Roxb.			✓	✓	✓			
41 <i>Sphagneticola trilobata</i> (L.) Pruski*					✓	✓		✓

The names of the species are updated from the Catalogue of Life (<https://www.catalogueoflife.org>)

\*Listed among 100 of the world's worst invasive alien species (Lowe et al. 2000)

### The most problematic invasive species: habitats and impact

The 20 most problematic invasive plants in South Asia occurred in a range of habitat types (Table 3). The highest numbers were found in disturbed habitats (13 species), followed by riverine habitats and grassland (11 each), forests (10), wetlands (8), and woodland (7). Forest edges, ponds, shrubland (3 each), and ditches (2) harbour the least problematic invasives. *Lantana camara* is a species that is widespread in the greatest number of habitats, i.e. disturbed sites, forests, forest edges, riverine habitats, pastures, and woodland (Fig. 4).



**Figure 4.** The occurrence of the most problematic invasive species in different habitat types. The presence of the species in a habitat is indicated by a blue cell. The classification of habitats of particular species is based on Weber (2017).

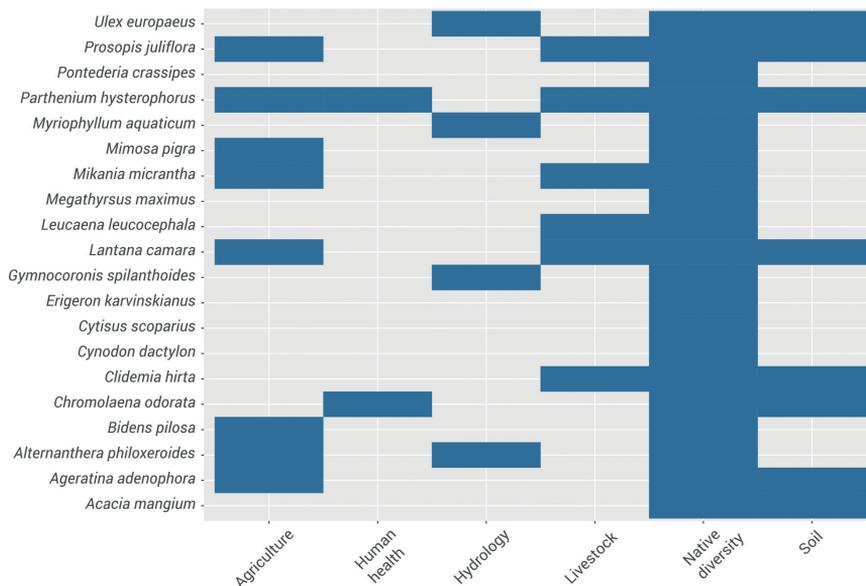
**Table 3.** The most problematic invasive plant species of South Asia with information on the region of origin, invaded range, growth form, introduction history, and negative impacts. Habitats: Cl, Cliff; Ds, Disturbed sites; Dws, Disturbed wet sites; Di, Ditches; Fo, Forest; Fo ed, Forest edges; Fp, Floodplain; Gr, Grassland; La, Lakes; Ps, Pastures; Pl, Plantation; Po, Ponds; Rd, Roadside; Rv, Riverine; Ro, Rock outcrops; Sr, Shrubland; Wd, Woodland; Wt, Wetland. The information was retrieved from Weber (2017), CABI (2022), and the Global Invasive Species Database (2022), where the original references can be found.

Species name, family, origin	Invasive range in SA	Invaded habitat	Growth form	Introduction history	Negative impacts
<i>Acacia mangium</i> ; Fabaceae; Australia, Papua New Guinea and Irian Jaya and the Maluku Islands of Indonesia	Bangladesh	Fo, Ds	Evergreen tree	Vector transmission, intentional as it is a commercially important species in SE Asia	<b>Native diversity:</b> threatens native diversity, prevents germination, outcompetes native species for water and light, and changes the fire regime. <b>Soil:</b> alters soil macrofauna; increases nitrogen availability.
<i>Ageratina adenophora</i> Asteraceae; Central Mexico	India, Nepal, Bhutan, Bangladesh	Fo, Rv, Gr, Ds	Perennial herb, subshrub	Seed contaminant, as an ornamental during the 1800s	<b>Native diversity:</b> eliminates native vegetation and prevents regeneration. <b>Agriculture:</b> inhibits germination and seedling growth of rice. <b>Soil:</b> allelopathy affects soil microbes.
<i>Alternanthera philoxeroides</i> Amaranthaceae; South America, Argentina	Bangladesh, India, Nepal, Sri Lanka	Wt, Dws	Aquatic perennial herb	Natural dispersal, accidentally introduced in ballast water, boats, vehicles, and by animals; intentionally as an ornamental and aquarium plant	<b>Native diversity:</b> replaces native species; competes with pastures. <b>Hydrology:</b> creates dense mat in water; affects floating aquatic plants; impairs water flow and light penetration; promotes sedimentation and flooding; disease vector; degrades aesthetic value. <b>Agriculture:</b> causes problems in rice fields.
<i>Bidens pilosa</i> Asteraceae; South and Central America	Bhutan, India, Nepal	Gr, Fo, Wt, Ds, Rd	Annual herb	Contaminant in crop seeds and agricultural products, intentionally for ornamental and agricultural purposes	<b>Native diversity:</b> eliminates native vegetation by suppressing germination; acts as a host and vector of harmful parasites; crosses with native and endemic species on Hawaii island; allelopathic. <b>Agriculture:</b> declines crop yields.
* <i>Chromolaena odorata</i> Asteraceae; Tropical Central and South America, from Mexico and the Caribbean to Brazil	India, Nepal, Sri Lanka, Bangladesh	Fo, Rv, Gr, Wd, Pl	Shrub	Introduced to the Calcutta Botanical Garden as an ornamental plant in the 19 <sup>th</sup> century, further movement by the military in World War II	<b>Native diversity:</b> eliminates native diversity; prevents the establishment of other species due to competition and allelopathy; increases fire hazards. <b>Human health:</b> skin complaints and asthma. <b>Soil:</b> change invertebrates' community.
* <i>Clidemia hirta</i> ; Melastomataceae; Central and South America, Caribbean islands	India, Sri Lanka	Fo, Ps	Evergreen shrub	Accidentally by people as an ornamental plant	<b>Native diversity:</b> reduces diversity and leads to the extinction of native species, and affects ecosystems. <b>Livestock:</b> poisonous to animals. <b>Soil:</b> affects soil water-holding capacity.
<i>Cynodon dactylon</i> ; Poaceae; Africa	India, Bhutan (Virtually present at every tropical and subtropical country)	Gr, Wd, Wt, Ro	Perennial herb	Unclear	<b>Native diversity:</b> alters ecosystem functioning, fire regimes, hydrological cycles, nutrient cycling, and community composition.
<i>Cytisus scoparius</i> ; Leguminosae; Europe	India	Gr, Rv, Fo, Ds	Perennial shrub	As an ornamental plant, horticulture species, movement of farm equipment	<b>Native diversity:</b> declines native plant diversity; alters nutrient cycling; affects wildlife; increases fire hazards; changes species number and composition; prevents reforestation.

Species name, family, origin	Invasive range in SA	Invaded habitat	Growth form	Introduction history	Negative impacts
<i>Erigeron karwinskianus</i> ; Asteraceae; Central America, Mexico	India, Sri Lanka, Bangladesh, Nepal	Gu, Cr, Fo ed, Dws, Ds	Perennial herb	As an ornamental plant in Botanical Garden in Sri Lanka, then in India as a commodity contaminant	<b>Native diversity:</b> replaces native plants, affects regeneration, replaces vulnerable species in the alpine regions, habitat alteration, damages ecosystem services.
<i>Gymnocoronis spilanthoides</i> ; Asteraceae; America from Mexico to Argentina	India	Wt, Po, Rv	Aquatic perennial herb	Introduced as an aquarium plant and as an ornamental pond plant	<b>Native diversity:</b> displaces native plants, degrades natural wetlands, affects birds, and affects natural and recreational beauty. <b>Hydrology:</b> floating mats impede water flow, decline its quality, reduce light and block channels, decrease oxygen level, and cause flooding by blocking streams and drainage.
* <i>Lantana camara</i> ; Verbenaceae; Neotropics	Nepal, India, Pakistan, Bhutan, Bangladesh, Sri Lanka, Maldives	Fo, Fo ed, Wd, Rv, Ps, Ds	Evergreen shrub	Ornamental shrub, cultivated as a hedge plant	<b>Native diversity:</b> removes native vegetation and affects productivity; threatens endemics, affects the regeneration of tree seedlings; increases fire hazards; changes bird assemblage; a host for pests and diseases; deteriorates habitats of wildlife. <b>Livestock:</b> poisonous to livestock. <b>Agriculture:</b> the invasion on cultivated lands led to the shift of villages. <b>Soil:</b> in the stands of lanana, the water absorption capacity of soil declines, which increases the risk of soil erosion.
* <i>Leucaena leucocephala</i> ; Leguminosae; Mexico	India, Pakistan, Sri Lanka, Maldives, Bangladesh	St, Rv, Wd, Ds	Evergreen shrub, tree	By the late 1880s, it was widespread throughout Asia, promoted by the development of agricultural and forestry	<b>Native diversity:</b> suppresses native vegetation; prevents regeneration of native trees; promotes the establishment of other invasive species; alters nutrient cycling and ecosystem services; threatens endemic species. <b>Livestock:</b> toxic to livestock if consumed in high quantities.
<i>Megathyrsus maximus</i> ; Poaceae; Africa	Sri Lanka	Gr, Wd, Ps, Rv, Ds	Perennial grass	Introduced as a fodder plant, contaminant to seeds	<b>Native diversity:</b> replaces native vegetation; increases fire hazards; displaces natural grassland; retards seedling growth; habitat deterioration; competes with native species.
* <i>Mikania micrantha</i> ; Asteraceae; Central and South America	Nepal, India, Sri Lanka, Bangladesh, Pakistan	Fo, Fo ed, Rv, St, Ds	Perennial vine	In 1918, this weed entered India during World War I to camouflage airfields	<b>Native diversity:</b> replaces native vegetation; decreases productivity in agriculture; prevents forest regeneration; shades other species; competes for water and releases allelochemicals which inhibit the germination of seeds; suppresses the growth and kills other species; in Chitwan National Park of Nepal <i>Bhimbans unicornis</i> is under threat due to its invasion. <b>Livestock:</b> decreases livestock production. <b>Agriculture:</b> the worst weed of tea in India and Nepal and of rubber in Sri Lanka.
* <i>Mimosa pigra</i> ; Fabaceae; Neotropics	Sri Lanka, Nepal	Wt, Fo, Rv, Ds	Evergreen shrub	Introduced as an ornamental and seed contaminant; in Sri Lanka it was noted in 1997	<b>Native diversity:</b> removes native diversity and affects regeneration; infests wetlands; interferes with irrigation system; affects electric power lines; deteriorates recreational value; transforms floodplains into species-poor scrub; makes area inaccessible to wildlife; affects grazing area. <b>Agriculture:</b> negative impacts in rice cultivation.

Species name, family, origin	Invasive range in SA	Invaded habitat	Growth form	Introduction history	Negative impacts
<i>Myriophyllum aquaticum</i> ; Holaragaceae; South America	Nepal	La, Po, Wt, Rv	Submersed aquatic perennial	Aquatic garden plant but escaped cultivation	<b>Native diversity:</b> native macrophytes are outcompeted, causes water deoxygenation. <b>Hydrology:</b> restricts water flow; affects fisheries and recreation value; suitable habitat for mosquitoes; alters physical and chemical properties of lakes.
<i>Parthenium hysterophorus</i> ; Asteraceae; Mexico, Central and South America	Bangladesh, Bhutan, India, Pakistan, Nepal	Gr, Sr, Ps, Ds	Annual herb	Accidental introduction in India as a contaminant to cereal grains	<b>Native diversity:</b> declines native biodiversity, degrades natural ecosystems, and changes wildlife habitat. <b>Soil:</b> changes soil chemistry by increasing soil nutrients and pH. <b>Agriculture:</b> reduces crop productivity; affects crop production. <b>Livestock:</b> removes grass species; allelopathic properties decline meat and milk quality. <b>Human health:</b> causes dermatitis.
* <i>Pontederia crassipes</i> ; Pontederiaceae; Tropical South America	India, Nepal, Sri Lanka, Maldives, Bangladesh	Wt, Po, Di	Floating aquatic	Water ornamental in botanical gardens, used in aquariums	<b>Native diversity:</b> thick mats cover the water surface, affect the ecosystem and water quality, interfere with water transport, halt fishing; light reduction kills macrophytes, alters temperature; reduces habitats for fishes and birds; clogs irrigation channels; reduces oxygen level and increases nitrogen level; allelopathic effects; affect hydroelectric plant; breeding site for disease-carrying insects.
<i>Prosopis juliflora</i> ; Leguminosae; Mexico, Central and northern South America	India, Pakistan, Sri Lanka	Gr, Rg, Wd, Fp, Ds	Evergreen shrub, tree	Introduced as a fodder and fuel species, occurred in the 19 <sup>th</sup> century in India, accidentally introduced to other countries	<b>Native diversity:</b> reduces native species diversity; affects wildlife movement; hybridization with others; blocks paths and makes them impenetrable; alters nutrient cycling. <b>Soil:</b> changes soil chemistry and soil microbial community; promotes soil erosion; lowers water tables. <b>Agriculture:</b> loss in agricultural productivity. <b>Livestock:</b> death of livestock due to its consumption.
* <i>Ulex europaeus</i> ; Leguminosae; Atlantic maritime regions	India, Sri Lanka	Gr, Wd, Rv, Cl, Ds	Evergreen shrub	Intentionally spread as a hedge plant, ornamental and forage plant, or as a contaminant	<b>Native diversity:</b> grows on forest edges; eliminates native vegetation and prevents regeneration; affects wildlife; increases fire hazards; removes pastoral vegetation; damages ecosystem services. <b>Soil:</b> acidifies soil and alters its condition by fixing nitrogen. <b>Hydrology:</b> hydrological conditions; habitat alteration.

All the most problematic invasive plants in South Asia affect native species diversity (Table 3). In addition, eight species are reported to reduce the productivity of agricultural fields and alter soil properties, hence directly affecting the economy (Fig. 5). *Clidemia hirta*, *Lantana camara*, *Leucaena leucocephala*, *Mikania micrantha*, *Parthenium hysterophorus*, and *Prosopis juliflora* were reported to affect livestock and their products. Species like *Alternanthera philoxeroides*, *Gymnocoronis spilanthoides*, *Myriophyllum aquaticum*, and *Ulex europaeus* are responsible for hydrological changes that subsequently affect aquatic ecosystems. Only two invasive plants (*Chromolaena odorata* and *Parthenium hysterophorus*) are reported to have an impact on human health (Fig. 5).



**Figure 5.** The impact of the most problematic invasive plants in South Asia classified into impact categories. The recorded impacts are indicated by blue cells. The information on impacts was taken from Weber (2017), CABI (2022) and Global Invasive Species Database (2022). See Table 3 for detailed description of impacts of particular species.

### Implemented management approaches for selected invasive species in South Asia

Only 17% of research papers focused on the management of invasive plants in South Asia, most of them on a single species (i.e. *Ageratina adenophora*, *Chromolaena odorata*, *Lantana camara*, *Mikania micrantha*, *Parthenium hysterophorus*, *Pontederia crassipes*; Sullivan et al. 2017; Raj et al. 2018; Poudel et al. 2019; Sharma et al. 2022). Physical or mechanical removal was the most widely used management method. Manual slashing, use of tractors, plowing, hand pulling, sickle weeding, repeated cutting, and burning were commonly applied. The physical methods were labour-intensive and effective only in a small area. Therefore, chemical methods, i.e. herbicides, were also used to limit the spread of invasive species (Tables 4, 5).

**Table 4.** List of the invasive plants with their management methods in South Asia. Public awareness (by informing local people about the impacts), biological control, competition (removing invasive species by competing with native species), drivers (by knowing factors that import invasive species), physical (manual removal), and uses (using plants as green manure or for bedding of livestock).

Species name	Awareness	Biological control	Competition	Chemicals	Drivers	Physical	Uses	References
<i>Ageratina adenophora</i>	✓	✓	✓	✓		✓	✓	Negi 2016; Balami and Thapa 2017; Poudel et al. 2019
<i>Chromolaena odorata</i>		✓	✓	✓		✓	✓	Saikia and Deka 2017; Sharma et al. 2022
<i>Clusia rosea</i>						✓	✓	Hitinayake et al. 2018
<i>Cyperus rotundus</i>				✓		✓		Raj et al. 2018
<i>Lantana camara</i>	✓	✓		✓	✓	✓	✓	Love et al. 2009; Kannan et al. 2014; Kannan et al. 2016; Raj et al. 2018
<i>Mikania micrantha</i>	✓	✓		✓		✓		Sapkota 2007; Paudel 2011; Khadka 2017; Sullivan et al. 2017; Aryal et al. 2018
<i>Mimosa pigra</i>			✓	✓		✓		Marambe et al. 2004
<i>Opuntia stricta</i>		✓						Shen et al. 2018
<i>Opuntia monacantha</i>		✓						Sushilkumar 2015
<i>Parthenium hysterophorus</i>		✓	✓	✓		✓	✓	Javid et al. 2006; Shrestha et al. 2011; Khan et al. 2014; Shabbir 2014; Shabbir et al. 2015; Rana et al. 2017; Dolai et al. 2019; Iqbal et al. 2020; Adnan et al. 2021; Weyl et al. 2021b
<i>Pontederia crassipes</i>		✓		✓		✓	✓	Kaffe et al. 2009; Mathiventhan et al. 2018; Raj et al. 2018; Gupta and Yadav 2020
<i>Prosopis juliflora</i>				✓	✓	✓		Ratnasekera 2016
<i>Ulex europaeus</i>		✓	✓	✓	✓	✓		Jayasekara et al. 2021
<i>Xanthium strumarium</i>		✓				✓		Shen et al. 2018
Number of species for which a given method is applied	3	10	5	10	3	12	6	

Biological control was used less often than physical methods. Biological control programs were implemented only in India and Pakistan. Due to open and porous international boundaries between India and other South-Asian countries, some of the biological agents like *Zygogramma bicolorata* have naturally reached Nepal, Bhutan, Pakistan, and Bangladesh. Although some biological control agents have established in South Asia, their impacts were not strong (Shrestha et al. 2022). Removing invasive species before flowering, sowing competitive species after their removal, raising awareness among people about the negative impacts, and identifying the factors responsible for the spread of invasive species are other ways to manage invasive species (Table 5).

**Table 5.** Specific control measures for selected invasive species with significant negative impacts on the environment. The information was retrieved from Weber (2017), CABI (2022), and Global Invasive Species Database (2022), where the original references can be found. \*Listed among 100 of the world’s worst invasive alien species.

Species name	Control measures
<i>Acacia mangium</i>	Uprooting seedlings, cutting trees, and use of herbicides retard growth; triclopyr herbicide mixed with oil used on cuttings.
<i>Ageratina adenophora</i>	Slashing, ploughing, and sowing of other species after removal; herbicides; stem gall fly ( <i>Procecidochares utilis</i> ), fungus ( <i>Passalora ageratinae</i> ).
<i>Alternanthera philoxeroides</i>	Repeated leaf removal; herbicides like metsulfuron-methyl, glyphosate, dichlobenil and a mixture of glyphosate and metsulfuron-methyl; biocontrol by flea beetle ( <i>Agasicles hygrophila</i> ) successful in Australia.
<i>Bidens pilosa</i>	Persistent mowing and hand pulling, prevent germination by mulch; herbicides such as glyphosate-trimesium, oxyfluorfen, atrazine, 2,4-D glyphosate, pendimethalin, metribuzin, diuron, paraquat, nicosulfuron and simazine.
* <i>Chromolaena odorata</i>	Manual slashing, use of tractors to remove as hand pulling is labour intensive; repeated cutting and burning; chemicals 2,4-D, ester, picloram, imazapyr or 2,4,5-T applied at the seedling stage; triclopyr is the most effective.
* <i>Clidemia hirta</i>	Hand pulling, less soil disturbance, and cuts treated with triclopyr and glyphosate are effective.
<i>Cynodon dactylon</i>	Dug out and remove all rhizomes and stolons; infestation can be controlled by covering with plastic and applying paraquat or glyphosate.
<i>Cytisus scoparius</i>	Slashing, less soil disturbance, pulling out, goats and rabbits stunt growth and prevent regeneration; planting tall and competitive plants may contribute to reducing growth; use of chemicals like picloram, triclopyr, glyphosate, fluroxypyr, and metsulfuron-methyl.
<i>Erigeron karvinskianus</i>	Avoid soil disturbance; herbicide glyphosate, hexazinone, tebuthiuron.
<i>Gymnocoronis spilanthoides</i>	Mechanical removal and hand pulling lead to further spread; herbicides are effective only on the upper part; following herbicide application, the removal with machinery can be effective; dry and burn.
* <i>Lantana camara</i>	Mechanical clearing and hand pulling suitable for small areas; periodic burning; cleared areas should be revegetated; use of herbicides- 2,4,-D, MCPA, dicamba, triclopyr, glyphosate or picloram on cuts; well established biological agents: <i>Uroplata girardi</i> , <i>Ophiomyia camarae</i> , <i>Aconophora compressa</i> ; integrated approaches are recommended; in India, the control by spraying glyphosate on regenerated growth was effective.
* <i>Leucaena leucocephala</i>	Grazing by goats; solarization was found effective in killing all plants and seeds; pulling out roots and shading leads to seedling mortality; treating of cutting with picloram; cutting stems and treating them with diesel and other chemicals.
<i>Megathyrsus maximus</i>	Pulling out, heavy grazing; herbicides glyphosate prevent new growth; pathogens like <i>Drechslera gigantea</i> , <i>Exserohilum rostratum</i> , and <i>E. longirostratum</i> are highly effective.
* <i>Mikania micrantha</i>	Sickle weeding and uprooting prior to seed maturity; slashing or repeated cut from the ground; herbicide like paraquat and 2,4-D amine, glyphosate + picloram; parasitic plant <i>Cuscuta campestris</i> suppresses its growth; rust fungus ( <i>Puccinia spegazzinii</i> ); increasing shade in forests makes the habitat unsuitable for its growth; potential biological control: <i>Liothrips mikaniae</i> .
* <i>Mimosa pigra</i>	Complete digging out; killed by cutting at a depth of 10 cm; slashing and burning with the use of herbicides picloram, hexazinone, dicamba, triclopyr, linuron, and glyphosate; biological control <i>Nesaecepeida infuscata</i> released in Australia; restriction of the movement of vehicles, soil, and sand from infested areas; integrated approaches are beneficial.
<i>Myriophyllum aquaticum</i>	Biomass removal; cleaning boats; herbicides 2,4-D, diquat, or fluridone can be effective when plants are young; in South Africa, biological control by <i>Lysathia</i> was found effective.
<i>Parthenium hysterophorus</i>	Manual uprooting before flowering; mowing, slashing, plowing; herbicides 2,4-D, picloram and hexazinone; biocontrol agents: the leaf-feeding beetle <i>Zygogramma bicolorata</i> , the stem-galling moth <i>Epiblema strenuana</i> , the stem-boring beetle <i>Listronotus setosipennis</i> , and the seed-feeding weevil <i>Smicronyx lutulentus</i> .
* <i>Pontederia crassipes</i>	Physical or mechanical removal by machine can stop its spread, reduce the nutrient level in the water, chemicals 2,4-D, glyphosate; biological control by <i>Neochetina</i> weevils is effective; use of boom to control the movement of weed; utilization of biomass.
<i>Prosopis juliflora</i>	Control is highly expensive and unsuccessful; mixed mechanical and chemical control; hand pulling effective only on a small scale; stems cut at least 10 cm below ground will not resprout; herbicides: clopyralid, picloram, triclopyr, 2,4-D amine suppress the growth.
* <i>Ulex europaeus</i>	Hand pulling and repeated cutting; herbicides: glyphosate, picloram, triclopyr, and 2,4,5-T; prescribed burning; planting native trees and competitive grass suppress growth; intensive grazing by goats; biological control: <i>Sericothrips staphylinus</i> , <i>Excapion ulicis</i> , <i>Tetranychus linearius</i> ; integrated control reduces the spread.

## Discussion

Research focusing on plant invasions in South Asia has steadily increased in the last two decades, which corresponds to the increase worldwide (e.g. Pyšek and Richardson 2010; Ramírez-Albores et al. 2019; Muñoz-Mas et al. 2021). Still, despite the recent dynamic increase in research effort, plant invasions in Asia, especially in its tropical part, remain greatly understudied compared to other continents, particularly North America and Europe (Foxcroft et al. 2017). Most plants were introduced to South Asia as ornamental species, followed by those introduced for agriculture and horticulture, as contaminants of seed and transport machinery, or as stowaways (Banerjee et al. 2021). Australia, New Zealand, and South Africa focus more on management, but Asia lags behind, still describing basic patterns (Pyšek and Richardson 2010; Hulme 2020). Moreover, research effort is uneven in Asia; for example, no literature exists for Afghanistan and the Maldives. The capacity of most Asian countries to combat emerging plant invasions is poor compared to the countries in North America, Western Europe, and Oceania (Early et al. 2016).

India is also known as one of the global hotspots of invasive alien species (Pyšek et al. 2017). India has the largest economy and makes up more than 70% of the South-Asian economy (<https://www.worldbank.org/en/region/sar/overview> retrieved on 5 Jan 2023). This country has the highest number of invasive and naturalized plant species among South-Asian countries (Inderjit et al. 2018). This is due to its large area and rapidly growing economy. However, the percentage of invasive and naturalized species is higher on the island of Maldives. This could be due to the continuous oceanic border, which increases the propagule pressure (Brock and Daehler 2022), and the greater vulnerability of islands to invasions (Pyšek et al. 2017; Moser et al. 2018). Afghanistan has the lowest number of invasive and naturalized species, but here, the most likely reason is inadequate research effort.

Our review revealed that there are at least 392 invasive plant species in South Asia. Among them, 41 species occur in at least three countries, and 20 species are considered the most problematic in terms of having negative ecological impacts (Weber 2017). This number is most likely to be higher as the impacts of many species have not yet been known. Species like *Mimosa diplotricha* and *Sphagneticola trilobata* are invasive in Nepal (Sharma et al. 2020; Shrestha et al. 2021) and India (Choudhury et al. 2016) but not mentioned in the GloNAF database. The impacts of invasive plants are of great concern in South Asia because the majority of people directly depend on natural resources. *Chromolaena odorata*, *Clidemia hirta*, *Lantana camara*, *Leucaena leucocephala*, *Mikania micrantha*, *Mimosa pigra*, *Pontederia crassipes*, *Sphagneticola trilobata*, and *Ulex europaeus* are among the 100 of the worlds' worst invasive species, and they are widespread in South Asia. Unfortunately, most impact studies focus on a single species and are carried out at a small geographic scale. To quantify the real impacts of invasive plants, studies should be carried out in open and extensive landscapes and under natural conditions.

## Studies on impacts of invasive plants in South Asia

In South Asia, research on the impacts of invasive species started only after 2001. Most of the studies have focused on the impacts of single invasive species such as *Pontederia crassipes*, *Lantana camara*, *Mikania micrantha*, and *Parthenium hysterophorus* (Kohli et al. 2006; Ahmed et al. 2007; Murphy et al. 2013; Rawat et al. 2019; Bhatta et al. 2020). The impacts of invasive plant species on native vegetation are reported from South Asia, and studies showed that invasive plants commonly reduced the richness, diversity, and evenness of native species (Thapa et al. 2016; Bhatta et al. 2020; Kumar and Garkoti 2021) and changed the species composition. However, studies focusing on impacts on socioeconomy, agriculture, health, and hydrology are not sufficiently represented. The particular findings from South Asia, reported in detail below, correspond to the global analyses by Vilà et al. (2011) and Pyšek et al. (2012).

## Impacts on native plant diversity in natural ecosystems

Plant invasions have serious impacts on the environment of Asia, including natural habitats. In forests, dense patches of invasive plants inhibit seedling growth by blocking sunlight and stimulating the growth of other alien plants (Dogra et al. 2009a, b; Rupasinghe and Gunaratne 2017). In the Himalayas, invasive species like *Ageratina adenophora* and *Lantana camara* are problematic in pine forests and riparian forests because they enhance the soil nutrient cycling in invaded microsites and spread rapidly (Parveen et al. 2011; Kumar et al. 2021). In Indian forests, *L. camara* has posed a threat by replacing native understorey vegetation and hindering tree regeneration (Kohli et al. 2006). Similarly, in Nepal's Bardia National Park, the invasion of *Lantana camara* has been responsible for over 50% reduction in native plant richness and diversity (Bhatta et al. 2020). Invasive species richness was reported to be inversely proportional to the tree canopy (Thapa et al. 2020); therefore, maintaining a closed tree canopy can prevent the invasion problem.

Invasion in grasslands suppressed palatable grasses and decreased their regeneration, threatening wildlife and making their habitat unsuitable (Akter and Zuberi 2009; Sullivan et al. 2017; Chhogvel and Kumar 2018). *Parthenium hysterophorus* is highly problematic in the grasslands of Nepal, India, and Pakistan (Javaid and Riaz 2012; Shrestha et al. 2015; Rokaya et al. 2020). The presence of invasive *Centaurea iberica* in the mountain grasslands of India suppressed native plant species diversity and changed their species composition (Reshi et al. 2008).

Thickets of invasive plants prevent the exchange of sunlight and heat, leading to poor oxygenation and the presence of carbonic and bicarbonic acids (Nguyen et al. 2015; Pandey et al. 2020). *Pontederia crassipes* is one of the world's worst invasive weeds, which alters the physicochemical properties of water (Basaula et al. 2021) and competes with native hydrophytes for oxygen (Rashid et al. 2014). It damages aquatic ecosystems and deteriorates their aesthetic value (Pathak et al. 2021) and was

reported to alter hydrological regimes and replace aquatic flora (Gupta and Yadav 2020; Pathak et al. 2021). Moreover, plant invasions in wetlands negatively affect crop production by hampering irrigation systems, blocking fishing areas, declining fish production, setting barriers to boating, and altering the water cycle (Keller et al. 2018; Pathak et al. 2021).

## Impacts on agriculture, soil, and human health

Plant invasions decrease agricultural productivity by reducing nutrient levels in the soil (Yakandawala and Yakandawala 2011; Painsi et al. 2016; Chhogyel and Kumar 2018; Chhogyel et al. 2021). The fluctuation in agricultural production affects national economies and threatens food security (Kohli et al. 2006). Economic costs due to invasive species in agriculture are estimated in some countries, such as India and Pakistan (Diagne et al. 2020), but the estimates are still missing for other countries, for example Bangladesh (Mukul et al. 2020). The increased impact of *Phenacoccus solenopsis* on the cotton yield of India caused a loss of about US\$ 1.217 billion and is forecasted to increase in coming years (InvaCost; Diagne et al. 2020). *Ageratum conyzoides*, *Ageratum houstonianum*, and *Parthenium hysterophorus* cause problems in the agricultural fields of South Asia (Kohli et al. 2006; Shrestha et al. 2019), and their impacts are reported in Nepal and Pakistan (Javaid and Riaz 2012; Shrestha et al. 2015; Rokaya et al. 2020). *Ageratum conyzoides* invading agricultural fields has caused a decline in crop productivity (Kohli et al. 2006; Shrestha et al. 2019; Shah et al. 2020). The impacts of invasive species are more pronounced in developing countries because local people depend more on agriculture, fisheries, and forestry (Mungi et al. 2018; Shah et al. 2020).

Some invasive plants produce allelopathic substances that affect plant diversity as well as soil microbial diversity by leaching allelochemicals into the soil (Inderjit et al. 2011; Thapa et al. 2020). Research has shown that invaded soils have high microbial biomass and rapid litter decomposition, which increases the availability of nutrients and, as a result, invasive species grow rapidly (Ahmad et al. 2019; Zhao et al. 2019; Kumar et al. 2021). These toxic chemicals help invasive species establish and spread rapidly (Kumar and Garkoti 2022).

Besides declining native plant diversity and changing ecosystem properties, invasive plants cause several diseases to humans and livestock in South Asia (Kumar and Prasad 2014; Rashid et al. 2014; Negi 2016). *Parthenium hysterophorus* is known to have negative impacts on human health, causing skin allergy, rhinitis, and irritation to the eyes (Kohli et al. 2006; Adkins and Shabbir 2014; Shrestha et al. 2015; Chhogyel et al. 2021). Due to direct exposure to invasive plants, health problems are also greater in developing countries. Several other species, like *Ageratum houstonianum* and *Mimosa diplotricha*, negatively affect human health and livestock conditions (Shrestha et al. 2019; Sharma et al. 2020), but there is very little research in this respect. On the other hand, some invasive plant species are used in traditional medicine as antimicrobial, antiseptic, and blood coagulants (Negi 2016).

## Management

Despite the recent increase in the number of published studies, research on the management of invasive plants in South Asia is still insufficient. Chemical, physical, and mechanical removal of invasive species are the most common practices in South-Asian countries (Raj et al. 2018). There are attempts to manage invasive species by physical removal with the participation of the local people (Sullivan and York 2021), which is labour intensive. For instance, the management of *Pontederia crassipes* by utilizing its biomass for various purposes has been adopted but was unsuccessful because of the absence of continuous funding (Patel 2012).

Experiences from other parts of the world show that control of invasive plants by physical and chemical methods is expensive and needs continuous long-term effort. Great Britain spent about ~£90 million annually on chemicals for controlling invasive weeds in agricultural land (Williams et al. 2010). On the other hand, biological control is the most effective and sustainable method to control invasive species because once established, it perpetuates itself and does not need continuous financial inputs for management (Clewley et al. 2012). Most of the countries which are successful in the eradication of invasive plants have adopted biological methods. For example, *Azolla filiculoides* have been controlled for over a decade in South Africa by a North American frond-feeding weevil, *Stenoplemus rufinasus* (Hill et al. 2008). In Australia, nine insects and two fungal pathogens are used as biological control agents against *Parthenium hysterophorus* (Dhileepan et al. 2019). Unfortunately, biological control is in the early stage and poorly developed in South Asia due to the high initial cost and long time required for screening. However, some biological control agents for *Ageratina adenophora*, *Chromolaena odorata*, *Lantana camara*, *Mikania micrantha*, *Parthenium hysterophorus*, and *Pontederia crassipes* were introduced to South Asia (Dhileepan and Senaratne 2009; Poudel et al. 2020; Shrestha et al. 2022). In Papua New Guinea, a gall fly *Cecidochares connexa* was found to successfully control the populations of invasive *Chromolaena odorata* (Day et al. 2013). In South Africa and some neighbouring countries, the flowering galling mite *Aceria lantanae* reduced the flower production of *Lantana camara* by up to 97% (Simelane et al. 2021).

Most alien species were introduced to South Asia for ornamental purposes, soil improvement, or as a fodder crop for animal husbandry; some were introduced as contaminants (Tiwari et al. 2005; Ekanayake et al. 2020). For instance, *Lantana camara* and *Pontederia crassipes* were introduced to botanical gardens in India as ornamentals (Kohli et al. 2006). Similarly, *Spermacoce alata* seeds entered Nepal along with the seeds of forage plants distributed to farmers (Shrestha 2016). There is abundant evidence showing that disturbance increases resource availability, making a plant community susceptible to invasion (Davis et al. 2000; Dogra et al. 2009b). Forest edges, agricultural land, grasslands, fallow land, roadside vegetation, and wetlands are susceptible to invasion as they feature higher levels of disturbance (Biswas et al. 2007; Shrestha and Dangol 2014; Rupasinghe and Gunaratne 2017). Moreover, lack of natural enemies,

physical disturbance, and open forest canopies are also among the causes of the success of invasive plants (Mandal and Joshi 2014). Passenger air travel is considered one of the introduction vectors in South Asia (Early et al. 2016). Identifying the major drivers and pathways of plant invasions is important for their management.

Species like *Lantana camara* are very widespread and difficult to eradicate by mechanical, chemical, and biological methods (Love et al. 2009). In South Asia, the eradication of *L. camara* is nearly impossible, but the negative impacts could be reduced through management. Additionally, efforts should be made to prevent invasions in new areas. In Pakistan, chemicals like glyphosate and metribuzin are effective in controlling *Parthenium hysterophorus* when treated in a rosette stage (Khan et al. 2012). Herbicide treatment and competitive plants are also used in Pakistan to manage this species (Adnan et al. 2021). The chemical method is effective but not recommended because of its detrimental effects on other biota (Love et al. 2009; Rana et al. 2017). Allelopathic evaluation of invasive plants is important for the biological control of *P. hysterophorus* (Shinwari et al. 2013). Biological control using *Zygogramma bicolorata* has successfully retarded the growth of invasive *P. hysterophorus* by defoliating the plants (Shrestha et al. 2011; Shabbir et al. 2015; Weyl et al. 2021a, b). In addition to this, winter rust, *Puccinia abrupta* var. *partheniicola* is also reported to control *P. hysterophorus* by damaging leaf tissues (Iqbal et al. 2020; Maharjan et al. 2020; Weyl et al. 2021a). Australia has deliberately released this biological control, but countries like Nepal, India, Pakistan, and China have reported this rust to occur without intentional introduction (Iqbal et al. 2020). Laboratory experiments with *Listronotus setosipennis* in Pakistan have shown that this weevil is specific to *P. hysterophorus* (Weyl et al. 2021a). Similarly, *Procecidochares utilis* causes stem galling and suppresses the growth of *Ageratina adenophora* (Poudel et al. 2019). However, its effectiveness is low in the Himalayan region (Poudel et al. 2019). The main benefit of biological control methods is that they perpetuate by themselves but need rigorous research on host-ranging tests before releasing them in nature (Pateron et al. 2021). Countries have implemented different ways of eradication and management of invasive species, but biological control is still in its early stages in South Asia.

Australia and New Zealand have successfully managed some of the problematic invasive alien species that are also widespread in South Asia by focusing on prevention (Raj et al. 2018). In Australia, every dollar spent on the prevention of invasion benefits \$25.60–38.30 (Sinden et al. 2004). Countries of Asia should adopt integrated methods of biological and chemical control, along with making use of competition with native plants, to effectively manage already established invasive plants (Shabbir 2014; Shabbir et al. 2015); identifying competitive native species and actively planting them can help in effective management (Khan et al. 2014; Balami and Thapa 2017). Moreover, the identification of dispersal pathways, high biosecurity, local community participation, and awareness among locals play a vital role in limiting the spread of invasive species (Kannan et al. 2016; Shrestha 2019). Another option could be using invasive species for biogas, firewood, and biofertilizer production, such as *Pontederia crassipes* (Kaffe et al. 2009; Raj et al. 2018). However, it is essential to be cautious in order not to unintentionally promote the invasive species.

## Conclusions: management recommendations

South Asia harbours a substantial proportion of global biodiversity, making it imperative to exert every possible effort in safeguarding it against current and potential future plant invasions. The region is part of a biodiversity hotspot area, yet the impact of invasive species is poorly understood. In this paper, we assess the most problematic invasive plant species in South Asia, their impacts, and management. There is no information about the effectiveness of management and policies adopted in South Asia. We show that South Asia still focuses on inventories and descriptive approaches, whereas the impacts of invasive species on the economy, hydrology, and human health are little explored and identified only for a few invasive species. Ecosystem impacts are also understudied; for example, how invasive plants affect ecological processes such as productivity, nutrient dynamics, and pollination have been poorly covered. Thus, by identifying the less explored research areas with regard to the most abundant and problematic invasive species in South Asia, this review contributes to bridging the data gap for global databases and identifies the priority areas for future research. There is an urgent need to quantify the impacts of all widespread and problematic species in South Asia, which is crucial for allocating resources for management. The management should prioritize invasive species with the highest environmental impacts and regions that are suffering the greatest loss.

Biological control is the most effective and sustainable way of retarding the spread of invasive species, but unfortunately, research on biological control is not adequate in South Asia. Our review suggests that research on biological agents should be increased, and community awareness is needed to make the management effective. It is important to recognize that the implementation of biocontrol measures can leverage insights from studies conducted in other regions, underlining the essential need to prioritize specific targets for effective biocontrol strategies.

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## Supplementary material I

### 372 papers were used for the analysis

Authors: Suneeta Bhatta, Bharat Babu Shrestha, Petr Pyšek

Data type: xlsx

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# Mining biodiversity databases establishes a global baseline of cosmopolitan Insecta mOTUs: a case study on Platygastroidea (Hymenoptera) with consequences for biological control programs

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## Abstract

In the past decade, several species of platygastroid wasps were found to be adventive in North America and Europe while under evaluation in quarantine as biological control agents of invasive pests. The scope and relative risk of this phenomenon is not fully known, but it is clearly a trend with implications for classical biological control. As a means of assessing the issue and to provide a global baseline, we implemented a data-mining approach with DNA sequences in the Barcode of Life Database, yielding 201 platygastroid BINs with intercontinental and island distributions. At least fifty-five BINs displayed exact COI barcode matches across continents, with many more BINs scored as inconclusive due to sequence length variation. These intercontinental and island BINs include biocontrol agents known to be adventive, as well as many species identified only to genus with uncertain geographic origins. We provide 2,500 identifications for platygastroid BOLD BINs, 88% to genus, to encourage additional research on this distributional phenomenon. The intercontinental BOLD BINs were compared to literature records and GBIF occurrences of cosmopolitan species to identify gaps and discordance across data sources. Smaller COI barcode datasets from localities in Florida and Germany, including topotypical specimens, revealed more intercontinental matches. We analyzed COI sequences in BOLD for the entirety of Insecta and Araneae to assess

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this phenomenon more broadly and because these taxa contain many hosts for platygastroid wasps. This method revealed that the intercontinental distribution phenomenon is widespread with implications for assessing biological diversity, taxonomic methodology and regulatory frameworks.

### Keywords

adventive species, biological control, biodiversity databases, COI barcoding

## Introduction

Human-mediated movement of insect pests is a well-known phenomenon, and mitigation attempts often include classical biological control. Relatively recent invasive stink bugs (Hemiptera, Pentatomoidea) in the United States include the kudzu bug (*Megacopta cribraria* Fab.), the brown marmorated stink bug (*Halyomorpha halys* (Stål)) and bagrada bug (*Bagrada hilaris* Burmeister); management efforts for all of them included classical biological control using egg parasitoids in the family Scelionidae (Hymenoptera, Platygastroidea). In each case, the biological control agent in quarantine was found to be adventive prior to approved release, circumventing regulatory processes and establishing expanded geographic ranges without oversight (Gardner et al. 2013; Talamas et al. 2015; Milnes et al. 2016; Ganjisaffar et al. 2018; Servick 2018; Hogg et al. 2021; Talamas et al. 2021). In the case of *Trissolcus japonicus* (Ashmead) attacking *H. halys*, this resulted in an immediate shift from determining if the parasitoids could be released, to tracking their movement and ecological effect (Buffington et al. 2018). This phenomenon, in which parasitoids are found in association with recently adventive pests, has been documented in Mexico, Chile and throughout Europe (Sabbatini-Peverieri et al. 2018; Lomeli-Flores et al. 2019; Stahl et al. 2019; Rojas-Gálvez et al. 2021; Dieckhoff et al. 2021) and is not limited to biological control agents that are waiting to be deliberately introduced. In the United States, two species that parasitize stink bug eggs, *Psix tunetanus* and *Ps. striaticiceps*, are known to be adventive (Johnson and Masner 1985; Birkmire et al. 2021), but were not imported into quarantine as part of a biological control program.

The prevalence of unintended introductions remains largely unevaluated and the numerous detections of scelionid parasitoids of stink bugs is probably a reflection of the attention given to these organisms. DeBach and Rosen (1991) estimated that forty-three percent of exotic beneficial arthropods in the United States arrived by accident. Sporadic appearance of biological control agents targeting invasive pests in new environments may be considered “fortuitous” (DeBach 1971). However, by definition, the consequences for native, non-target organisms remain unknown pending post hoc analysis. Tabulating the number of unintentionally introduced platygastroid wasps is even more problematic. An accurate estimate may be impossible to generate at present because taxonomic preparedness (Wheeler and Cracraft 1997) is generally lacking in hyper-diverse, economically important insect groups. Popovici et al. (2018) documented ‘tramp’ species in three platygastroid genera, all of which were identified

morphologically. This approach requires intimate knowledge of the world fauna and access to a cosmopolitan representation of specimens. Even when unintended introductions are detected relatively quickly (e.g., *Trissolcus japonicus*), verification of the species identity may require significant research and most cases are unlikely to be investigated extensively. As of 2021, twenty-nine platygastroid wasp species were considered introduced or invasive in the United States (Simpson et al. 2021) and we consider this to be an underestimate based on recent research (Talamas et al. 2021; Birkmire et al. 2021). Only eight introduced platygastroid species are documented in Europe and three of these were deliberate releases (Katsanevakis et al. 2015).

We suspect that unintended introductions of platygastroids will be increasingly detected as ‘dark taxa’ (Page 2016) in sequencing databases. The aptly named *Gryon aetherium* Talamas (Talamas et al. 2021) perfectly illustrates an instance where the range expansion of a ‘dark taxon’ was first detected by COI barcode matches and its classification and taxonomy were established subsequently. *Gryon aetherium* is a parasitoid of the pestiferous *Bagrada* bug (*B. hilaris*), which is found from South Africa to India. Given the size of this geographic range, hundreds of *Gryon* holotype specimens and descriptions had to be examined in detail before the species could be confidently described as new. The urgency increased as adventive populations were found in both North and South America and it was only with years of preceding work that the adventive populations in the United States and Chile could be reported with a species name - concurrent with the description of *G. aetherium* (Lomeli-Flores et al. 2019; Hogg et al. 2021; Rojas-Gálvez et al. 2021; Talamas et al. 2021). The potential for sequence databases to reveal adventive arthropod populations is significant, but limited by the metadata and level of identification associated with the sequences. For Platygastroidea, the Barcode of Life Database (BOLD; Ratnasingham and Hebert (2007)) contains nearly 140,000 scelionid and platygastroid sequence records as of October 2022. Most of these were bulk-sequenced as part of national initiatives and did not receive thorough taxonomic treatments. A total of 128,000 of these BOLD records are unidentified at the species level, whereas 108,000 are unidentified at the genus level. Accordingly, the number of platygastroid dark taxa potentially present in BOLD is staggering and association with existing names by comparison to type material, made by expert taxonomists, is a gigantic scientific effort.

In this contribution, we employed multiple research tracks to address the question of how many species of Platygastroidea may be moving over intercontinental distances while eluding detection. (1) We used existing BOLD infrastructure to identify platygastroid BINs (Barcode Index Numbers; database calculated clusters of highly similar sequences used to approximate species) that spanned large geographic areas. (2) Intercontinental platygastroid BINS were identified to genus. To ameliorate taxonomic impediments in the database and facilitate future research, we identified one fifth of all platygastroid BINs in BOLD to genus. (3) For each platygastroid genus with an intercontinental BIN, we compared the results of our data-mining approach with distributional data from taxonomic literature and the Global Biodiversity Information Facility (GBIF). (4) We incorporated data from two geographically disparate COI barcoding

projects that share the goal of reliably attaching species names to DNA sequences. The first was a year-long insect trapping effort in Jacksonville, Florida, USA, a type locality for many platygastroids described at the turn of the 20<sup>th</sup> century. Importantly, traps were operated near the Port of Jacksonville, a potential site of entry for exotic species. The second was the German Barcode of Life III: Dark Taxa, which seeks to enhance the size and quality of the German DNA barcode reference library and includes collecting localities from which platygastroids were described in the 19<sup>th</sup> century. By generating COI barcodes from toptotypical specimens and comparing these specimens to primary types as part of the identification, we made the most reliable association possible between a DNA sequence and a historical name, apart from using DNA from primary types directly. Additionally, this is the most feasible means to infer directionality of movement for species that appear to have adventive distributions. (5) We implemented our data-mining protocol for Insecta and Araneae for two reasons. First, platygastroids parasitize a taxonomically diverse array of insect and spider hosts and data about their distributions might inform our results for Platygastridae. Second, the phenomenon of intercontinental distributions of arthropods more broadly is worthy of attention, and we sought to demonstrate the scalability of our approach.

## Materials and methods

### BOLD data-mining for Platygastridae

Beginning in October 2022, BOLD v.4 (Ratnasingham and Hebert 2007) was manually datamined for Platygastridae BINs with intercontinental distributions. Our search terms for Platygastridae followed the revised classification of Chen et al. (2021), which recognized Geoscelionidae, Janzenellidae, Neuroscelionidae, Nixonidae, Platygastridae, Scelionidae and Sparasionidae as the extant families. The BOLD BIN database portal was queried with the following family and genus level terms: “Geoscelionidae”, “Janzenellidae”, “Neuroscelionidae”, “Nixonidae”, “Platygastridae”, “Scelionidae”, “Sparasionidae”, “*Huddlestonium*”, “*Plaumannion*”, “*Janzenella*”, “*Neuroscelio*”, “*Nixonia*”, “*Archaeoteleia*”, “*Mexon*”, “*Listron*”, “*Sceliomorpha*” and “*Sparasion*”. The resulting BINs were then assessed for the number of countries in which they occurred. BINs with multiple countries were further scrutinized. If the countries were on different continents or otherwise spanned large distances (e.g., Pacific islands and the mainland), then the BIN was included in the dataset. A set of information was recorded and downloaded for each intercontinental BIN: BOLD BIN, BOLD taxonomy lowest level, maximum intra-BIN p-distance, distance to nearest neighbor BIN, the BIN fasta file of COI sequences and the Darwin Core (Wieczorek et al. 2012) BIN metadata text file (10.5281/zenodo.7930011; 10.5281/zenodo.7930274).

In February 2023, the dataset was expanded using an automated scripting approach (10.5281/zenodo.7930407). The Darwin Core data files for “Insecta” and “Araneae” were downloaded from BOLD. The Insecta files were downloaded on 3 January 2023.

The Araneae files were downloaded on 16 March 2023. The Insecta file was examined for all the categories listed under the field “country”. The country fields were assigned to continents or island categories (Suppl. material 1). Continent assignments largely followed the United Nations Statistics Division (2023) designations, except in a few edge cases where changes were made to follow the borders of zoogeographic regions more closely. Edge cases were assigned to groups based upon the closest alignment of political boundaries and biogeographical barriers. To separate the Asian and European continents, Russia was divided along provincial borders that most closely continue the line of the Ural Mountains southward. Turkey, Georgia, Armenia and Azerbaijan were included in Europe to avoid dividing the Caucasus region. Island nations sufficiently distant from continents were placed into four categories based on oceanic region. The Indonesian provinces of Papua and West Papua were included in the Australian region to avoid dividing the island of New Guinea. Solomon Islands, New Caledonia and New Zealand were grouped with Australia.

### DNA sequence alignment, tree building and match scoring

Each BIN in the intercontinental and island platygastroid dataset was individually analyzed to determine whether they contained exact COI barcode matches across large distances. Each BIN’s COI fasta file was aligned using the default settings of MUSCLE (Edgar 2004) as implemented in MEGA7 (Kumar et al. 2016). In MEGA7, these individual BIN alignments were used to build neighbor-joining trees (when there were more than two terminal taxa) and distance matrices. Analysis settings were identical for both approaches: the data were labelled as protein-coding nucleotide sequences with the invertebrate mitochondrial genetic code, p-distance set as the method/model, substitutions included transitions and transversions, rates among sites were uniform and missing data treatment was set to partial deletion with a 95 percent site coverage cut-off. P-distance calculations involving  $\leq 255$  terminal taxa were exported as matrices. P-distance calculations involving  $> 255$  terminal taxa were exported as pairwise columns. Neighbor-joining trees were exported as Newick tree files and uploaded into the online annotation portal of Interactive Tree of Life (iTOL) v.5 (Letunic and Bork 2021).

Trees were viewed in iTOL to determine individual sequence membership in haplotype clusters. Apparent haplotype matches were then examined in the p-distance calculation files for confirmation. The geographic distribution of exact matches was evaluated by examining the specimen level metadata present in the BIN’s Darwin Core text file. Putative exact intercontinental matches were then validated in the underlying DNA alignment. This was necessary due to slight variation in the length of COI barcode sequences. If longer sequences in the alignments displayed polymorphisms toward either the 5’ or 3’ ends and the putative intercontinental matches lacked these flanking data, then the matches were considered inconclusive. Ambiguous DNA base pairs were ignored for considering exact matches.

Individual BIN alignments were ultimately combined into one fasta file for tree building and visualization. A species of *Periclistus* Förster (Hymenoptera, Proctotrupomorpha,

Cynipidae) with appropriate data coverage was selected to root subsequent analyses, based on the sister relationship of Cynipoidea to Platygastridae (Blaimer et al. 2023). Alignment of the combined intercontinental and island COI barcode dataset was performed in the MAFFT online service v.7 (Katoh et al. 2019) with the FFT-NS-1 setting. This alignment (Suppl. material 3) was used for neighbor-joining analysis in MEGA11 (Tamura et al. 2021) using the same tree building parameters described above. This tree topology (Suppl. material 4) was manipulated for viewing ease and annotated in iTOL v.5 (Letunic and Bork 2021) and FigTree v.1.4.4 (Rambaut 2018) (Suppl. material 5).

## Identification and BOLD digital morphology

During the above data-mining activity, it was noticed that a large proportion of platygastroid BINs were unidentified below the family level. A list of Platygastridae BINs was pulled from BOLD as candidates for identification using the provided digital morphology framework. Identifications were made, when possible, by comparison to images of primary type specimens provided by Talamas et al. (2017). Most Platygastridae BINs were sight-identified using the BOLD BIN images to family, sub-family, tribe or genus by taxonomic specialists Elijah Talamas, Zachary Lahey and Jessica Awad.

## GBIF data-mining and literature search

Genus level occurrence data (Table 1) were downloaded as Darwin Core Archives from the Global Biodiversity Information Facility (GBIF 2022) for all platygastroid genera identified in the intercontinental BIN dataset. These occurrence data were sorted by species, then country, to determine which species had records from more than one continent (Suppl. material 6). Relevant taxonomic revisions were searched to corroborate intercontinental taxa reported in GBIF and BOLD. For a total evidence analysis of Platygastridae, the GBIF, literature and BOLD datasets were combined to assess their continental and island connections. Species with records from multiple data types were only counted once and their geographic occurrences were rectified for analysis.

## Field work

Topotypes of platygastroid species described from Duval County, Florida, were collected between July 2018 and December 2021 in and around the Timucuan Ecological and Historic Preserve near the mouth of the St. Johns River. These collections were largely from Malaise traps that were placed at three different sites in the area. Collecting heads were provisioned with propylene glycol and wrapped with aluminum foil to prevent UV damage to the specimens. 3D printed yellow cylinder traps were experimentally deployed on the ground and suspended from overhanging branches during one sampling period and additional specimens were collected in yellow pan traps and by sweep netting around the trap sites. Bulk samples were returned to the laboratory and sorted under a Zeiss Discovery V8 Stereomicroscope. All platygastroid specimens were transferred to 95% ethanol and screened for matches to species described from that area.

**Table 1.** GBIF occurrence datasets used for comparison with the intercontinental BINs.

Family	Genus	GBIF Occurrence Dataset Citation
Platygastridae	<i>Allotropa</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.n3derh">https://doi.org/10.15468/dl.n3derh</a>
Platygastridae	<i>Amitus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.xjcn95">https://doi.org/10.15468/dl.xjcn95</a>
Platygastridae	<i>Amblyaspis</i>	GBIF.org (28 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.r8zptf">https://doi.org/10.15468/dl.r8zptf</a>
Platygastridae	<i>Aphanomerus</i>	GBIF.org (28 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.gw43u4">https://doi.org/10.15468/dl.gw43u4</a>
Platygastridae	<i>Euxestonotus</i>	GBIF.org (28 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.gu7djg">https://doi.org/10.15468/dl.gu7djg</a>
Platygastridae	<i>Fidiobia</i>	GBIF.org (14 February 2023) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.w478bm">https://doi.org/10.15468/dl.w478bm</a>
Platygastridae	<i>Leptacis</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.efh6t8">https://doi.org/10.15468/dl.efh6t8</a>
Platygastridae	<i>Metaclisis</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.qjwfp5">https://doi.org/10.15468/dl.qjwfp5</a>
Platygastridae	<i>Platygaster</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.d7n4wt">https://doi.org/10.15468/dl.d7n4wt</a>
Platygastridae	<i>Synopeas</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.ev6apy">https://doi.org/10.15468/dl.ev6apy</a>
Scelionidae	<i>Anteris</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.sjsua4">https://doi.org/10.15468/dl.sjsua4</a>
Scelionidae	<i>Aradophagus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.hbu7rt">https://doi.org/10.15468/dl.hbu7rt</a>
Scelionidae	<i>Baeoneurella</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.dw6d4e">https://doi.org/10.15468/dl.dw6d4e</a>
Scelionidae	<i>Baeus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.9hxwkx">https://doi.org/10.15468/dl.9hxwkx</a>
Scelionidae	<i>Baryconus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.qaraf2">https://doi.org/10.15468/dl.qaraf2</a>
Scelionidae	<i>Calliscelio</i>	GBIF.org (15 February 2023) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.bsua2e">https://doi.org/10.15468/dl.bsua2e</a>
Scelionidae	<i>Dicroscelio</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.67g54v">https://doi.org/10.15468/dl.67g54v</a>
Scelionidae	<i>Dyscritobaesus</i>	GBIF.org (20 February 2023) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.5mt6wa">https://doi.org/10.15468/dl.5mt6wa</a>
Scelionidae	<i>Gryon</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.tn8kne">https://doi.org/10.15468/dl.tn8kne</a>
Scelionidae	<i>Hadronotus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.9qz232">https://doi.org/10.15468/dl.9qz232</a>
Scelionidae	<i>Idris</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.h2q4j">https://doi.org/10.15468/dl.h2q4j</a>
Scelionidae	<i>Platyscelio</i>	GBIF.org (13 December 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.cprkz3">https://doi.org/10.15468/dl.cprkz3</a>
Scelionidae	<i>Psix</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.cc9rkg">https://doi.org/10.15468/dl.cc9rkg</a>
Scelionidae	<i>Telenomus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.32xjmc">https://doi.org/10.15468/dl.32xjmc</a>
Scelionidae	<i>Trimorus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.ytcg37">https://doi.org/10.15468/dl.ytcg37</a>
Scelionidae	<i>Trissolcus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.r4qbu5">https://doi.org/10.15468/dl.r4qbu5</a>
Scelionidae	<i>Xenomerus</i>	GBIF.org (31 October 2022) GBIF Occurrence Download <a href="https://doi.org/10.15468/dl.5vzkr6">https://doi.org/10.15468/dl.5vzkr6</a>

## COI barcoding of Florida specimens

DNA was non-destructively extracted from specimens using the Qiagen DNeasy Blood and Tissue Kit. Molecular voucher specimens were recovered and deposited at the Florida State Collection of Arthropods (Florida Department of Agriculture and Consumer Services – Division of Plant Industry; Gainesville, Florida). PCRs were conducted as 25 µl reactions using the KAPA HiFi HotStart Readymix Kit (Roche Diagnostics) per the manufacturer's recommended protocol. Oligonucleotide primers used for PCR and direct sequencing were the universal arthropod COI barcoding sets LCO1490/HCO2198 (Folmer et al. 1994) and LEP-F1/LEP-R1 (Hebert et al. 2004). PCR products were visually verified by gel electrophoresis and positive products were prepared for sequencing with the Qiagen QIAquick PCR purification kit. PCR products were bidirectionally sequenced utilizing BigDye Terminator v.3.1 chemistry on the Applied Biosystems SeqStudio platform. Sequence traces were trimmed and assembled into contigs in Sequencher 5.4.8 and Geneious Prime. New COI barcodes were uploaded to BOLD and GenBank (OQ561913–OQ561961) and assessed for their nearest matches.

## COI barcoding of German specimens

The German Barcode of Life III: Dark Taxa project (Hausmann et al. 2020) provided 34 platygastriine specimens matching 14 BINs with intercontinental distributions. These were selected for closer morphological examination and taxonomic analysis. Methods for trapping, non-destructive DNA extraction and COI sequencing follow Awad et al. (2021). Species partitioning was performed with ASAP (Puillandre et al. 2021) using the Jukes-Cantor model and default web server settings.

## Photography

Following non-destructive DNA extraction, voucher specimens were mounted and photographed with a Macropod imaging system consisting of a Canon EOS 6D Mark II camera body, EF 70–200 mm lens and 10× or 20× M Plan APO Mitutoyo objective lenses. Imaging software included Canon EOS Utility 3.14.30.4 and Helicon Focus Pro 7.7.5 for image stacking. Adobe Photoshop 23.2.2 was used for limited post-processing and addition of scale bars. Images were uploaded to BOLD with specimen metadata.

## BOLD data-mining for Insecta and Araneae

An R (R Core Team 2022) script was developed that created two outputs: 1) a list of BINs with intercontinental or mainland/island distributions and their associated metadata (e.g., taxonomic information) and 2) a list of BINs with intercontinental or mainland/island distributions and an individualized tally of long-distance occurrences. The platygastroid data were compared to our manually-extracted list. The taxonomic

spread in the Insecta file was summarized at the family and genus level, with special attention given to known host groups for platygastroids. Summary statistics were completed with R 4.2.2 (R Core Team 2022), using the `dplyr` (v.1.0.10; Wickham et al. (2022)), `data.table` (v.1.14.8; Dowe and Srinivasan (2023)), and `stringr` (v.1.4.1; Wickham (2022)) data manipulation packages. Data was visualized using the package `ggplot2` (v.3.4.1; Wickham (2016)). The full reproducible code is available in Supplementary materials (10.5281/zenodo.8380145).

A randomized 5% of the recovered Insecta and Araneae BINs were validated by manual examination in the BOLD BIN database (Suppl. materials 2, 19). Only the first page of returned search results (which contain order, species and country level distribution) were examined in the validation process. BINs with many different species identifications were scored as having minor taxonomic conflicts. BINs with multiple species or genus identifications were scored as having major taxonomic conflicts, except when it appeared that specific epithet discrepancies were due to varying genus classifications. The list of recovered Insecta and Araneae BINs was summarized at the family and genus level. BINs containing known host groups of Platyastroidea were similarly summarized. The Insecta and Platyastroidea occurrence datasets were transformed into pairwise matrices capturing their geographic distributions. Matrices (Suppl. materials 10, 12–16) were imported to the online version of Circos (Kryzwiniski et al. 2009) for visualization. Default settings were used in the online Circos viewer with the exception of the following: 1) labels segment set to large, 2) data filters intra-segment cells hidden, 3) row and column segments order set to col/row ratio, 4) row and column segments with normalized segment size set to remap segments size to 1000, 5) contribution tracks set to hide and no stroke, 6) ribbon caps completely disabled, 7) ratio layout enabled, 8) image format with no strokes and all tick labels hidden.

## Results

### BOLD data-mining for Platyastroidea

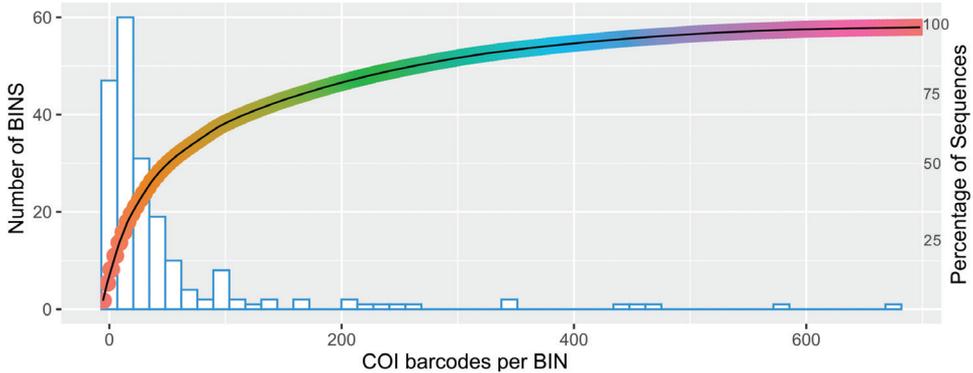
Two hundred and one platygastroid and scelionid BINs were found to have intercontinental and island distributions (Table 2, Suppl. material 11). No cases were detected in the other platygastroid families. Of these, 140 were identified only to the family or subfamily level in BOLD, sometimes incorrectly. We identified all but one of these 140 BINs to a lower taxonomic level; 130 of them were identified to genus or lower. The dataset contains 27 genera (10 Platygastridae genera; 17 Scelionidae genera) (Table 2, Suppl. material 11). *Telenomus* (69 BINs) and *Platygaster* (35 BINs) are the most represented genera in their respective families. Canada (101 points) and Germany (90 points) are the most represented country level occurrence points in the platygastroid BIN dataset (Table 2, Suppl. material 11), likely biased by the existence of nationally directed barcoding initiatives.

The minimum BIN size was two (necessary for a geographic match) and the largest, a *Platygaster* species, contained 677 COI sequences. Most BINs (180 of 201) contained

**Table 2.** Summary of platygastroid and host BINs displaying intercontinental and island distributions in BOLD. Data on host associations were derived from summaries in Masner and Huggert (1989) and Austin and Field (1997).

Taxon (parasitoid)	Intercontinental BINS (parasitoid)	Host taxon	Intercontinental genera (host)	Intercontinental BINS (host)
<b>Platygastridae</b>				
<i>Amblyaspis</i>	5	Cecidomyiidae	43	476
<i>Euxestonotus</i>	2			
<i>Leptacis</i>	7			
<i>Metaclisis</i>	1			
<i>Platygaster</i>	35			
<i>Synopeas</i>	13			
<i>Amitus</i>	1	Aleyrodidae	13	35
<i>Aphanomerus</i>	1	Flatidae	2	4
		Ricaniidae	3	4
<i>Allotropia</i>	1	Pseudococcidae	13	34
<i>Fidiobia</i>	2	Chrysomelidae	45	94
		Curculionidae	92	210
Platygastrinae	4			
<b>Scelionidae</b>				
<i>Anteris</i>	1			
<i>Aradophagus</i>	2	Theridiidae	20	52
<i>Baeoneurella</i>	1	Lygaeidae	7	20
<i>Baeus</i>	4	Araneae		
<i>Baryconus</i>	1	Tettigoniidae	8	11
<i>Calliscelio</i>	1	Gryllidae	6	8
<i>Dicroscelio</i>	2			
<i>Dyscritobaeus</i>	1			
<i>Gryon</i>	6	Heteroptera		
<i>Hadronotus</i>	5	Lepidoptera		
<i>Idris</i>	9	Araneae		
<i>Psix</i>	1	Heteroptera		
<i>Telenomus</i>	69	Heteroptera		
		Lepidoptera		
		Diptera		
		Neuroptera		
<i>Trissolcus</i>	11	Pentatomidae	21	27
<i>Trimorus</i>	7	Carabidae	48	105
<i>Xenomerus</i>	1			
Scelionidae	1			
Scelioninae	2			
Teleasinae	1			
Telenominae	3			

fewer than 100 COI sequences (Fig. 1, Suppl. material 11). Intra-BIN maximum p-distances reported by BOLD ranged from 0.00% to 5.42% (Suppl. material 11). Most BINs (157 of 201) in the platygastroid dataset had an intra-BIN maximum p-distance of less than 2.50% (Suppl. material 11). P-distance to the nearest-neighbor BIN was reported from all the platygastroid BINs. These p-distances ranged from 0.99% to 17.86% (Suppl. material 11). Twenty-eight BINs had larger intra-BIN p-distances



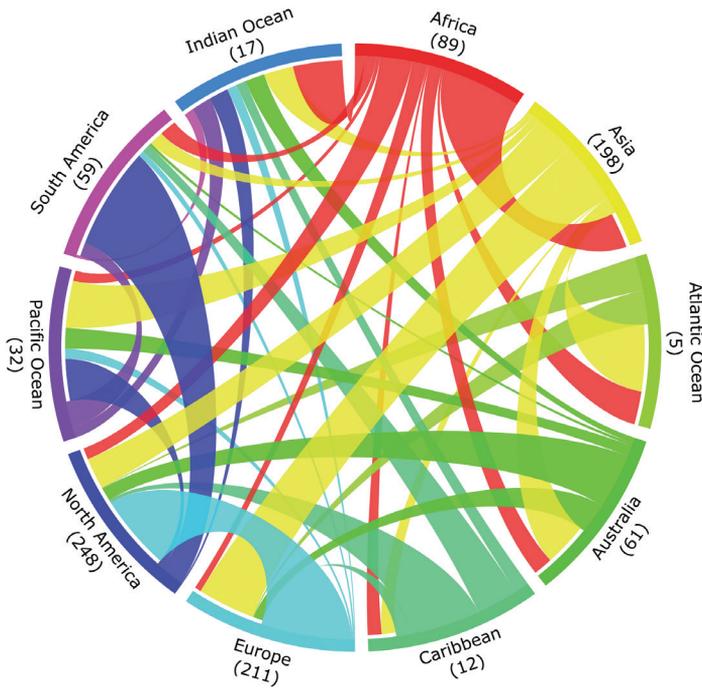
**Figure 1.** Pareto chart displaying the number of COI barcodes contained per BIN in the intercontinental and island platygastroid dataset.

than the p-distances to their nearest-neighbor BIN (Suppl. material 11). Fifty-five of the platygastroid BINs displayed exact COI matches across distant localities. These exact matches were discovered in *Amblyaspis* (2), *Amitus* (1), *Anteris* (1), *Aphanomerus* (1), *Dicroscelio* (1), *Euxestonotus* (2), *Fidiobia* (1), *Gryon* (2), *Idris* (3), *Leptacis* (2), *Platygaster* (9), *Psix* (1), *Synopeas* (5), *Telenomus* (18), *Trimorus* (1), *Trissolcus* (1), unidentified Platygastrinae (2), unidentified Scelioninae (1) and an unidentified Telenominae (1) (Suppl. material 11). Six BINs also included two exact intercontinental haplotype matches: *Euxestonotus* (1), *Gryon* (1), *Platygaster* (1), *Synopeas* (2) and *Telenomus* (1) (Suppl. material 11). An additional 28 BINs contained COI sequences that appeared to be exact matches, but were scored as inconclusive due to sequence length variation, precluding the most meaningful comparisons (Suppl. material 11).

In the total evidence Platygastroidea dataset, North America (248 connections), Europe (211 connections) and Asia (198 connections) were the most common connections (Fig. 2, Suppl. material 12). *Telenomus* and *Trissolcus* were the most represented scelionid genera across continents and islands. In Platygastridae, *Platygaster* and *Synopeas* were the most represented genera. The webs of geographic connection found in *Telenomus* and *Trissolcus* are more complex than those of *Platygaster* and *Synopeas* (Fig. 3, Suppl. materials 13, 14). For example, *Synopeas* and *Platygaster* lack any conclusive geographic data points in the Caribbean and Pacific Ocean and only one each in Africa. Europe and North America are the most connected continents in these genera, except for *Trissolcus* in which Asia and Europe are the most connected (Fig. 3, Suppl. materials 15, 16).

## DNA sequence alignment and tree building

The intercontinental and island BIN dataset contains 9,874 platygastroid COI barcode sequences. The MAFFT alignment was 669 base pairs wide after trimming excessive data from the 3' end of some barcodes. The alignment contains some gap regions due to varying COI amino acid phenotypes present among Platygastroidea (Talamas

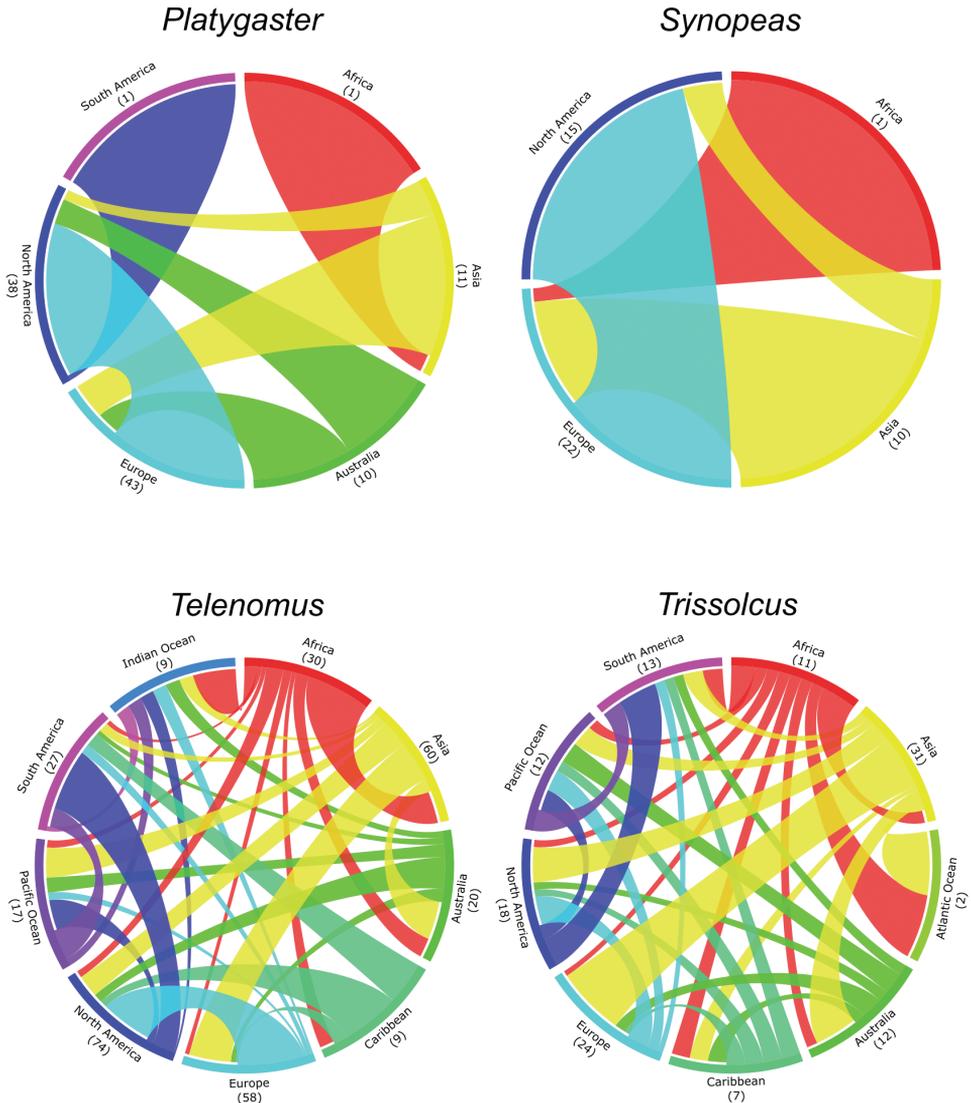


**Figure 2.** Circos plot displaying the relative contribution of continents and oceanic island groups to geographic connections in the combined platygastroid BOLD BIN, GBIF and literature dataset. Numbers in parentheses indicate the total number of connections to that geographic grouping.

et al. 2021). All positions with less than 95% site coverage were eliminated in the combined BIN p-distance neighbor-joining analysis; 520 positions were included in the final analysis. Terminal sequence clusters were collapsed and annotated to reflect their BIN assignment, revised or confirmed genus level identification and the number of sequences in that cluster (Fig. 4, Suppl. material 5). These terminal clusters corresponded unambiguously to their BIN assignment (i.e., they formed “monophyletic groups”), except in the case of *Telenomus* BOLD:AAU4881.

### Identification and BOLD digital morphology

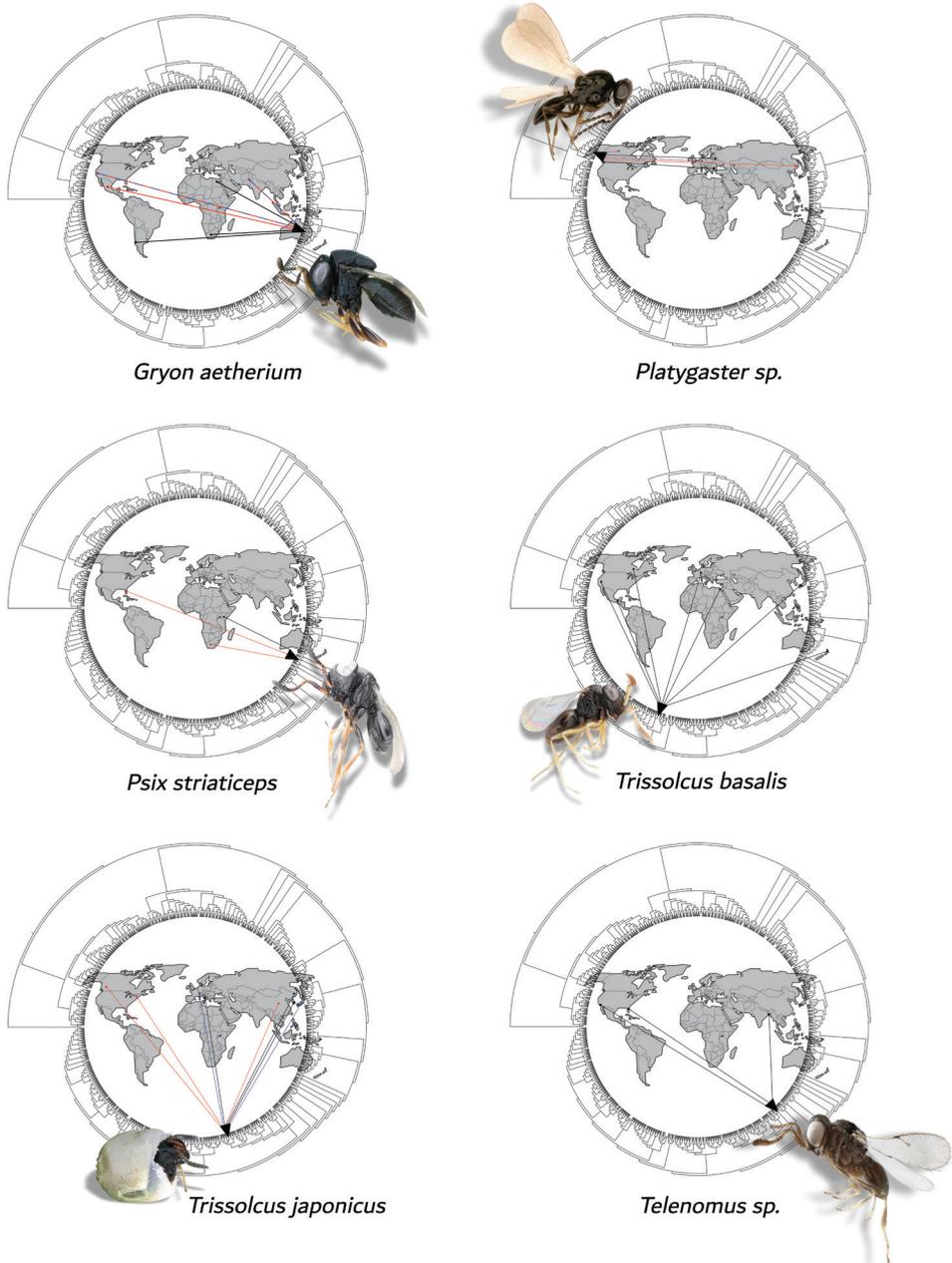
A total of 2,565 Platygastridae BINs were evaluated for their identification accuracy using the specimen images provided by BOLD (Suppl. material 17). Nearly all these BINs were correctly identified to superfamily (12 were misidentified to order or family). Updated subfamily classification was provided for 2,551 platygastroid BINs (Suppl. material 17). A total of 2,209 BINs had images of sufficient quality to add genus level identifications. Sixty-four platygastroid genera were present among these BINs (Suppl. material 17), of which eight represent the first DNA sequence data for the genus in GenBank or BOLD: *Euxestonotus*, *Gastrotrypes*, *Isocybus*, *Metaclisis*, *Parabaeus*, *Embioctonus*, *Styloteleia* and *Xenomerus*.



**Figure 3.** Circos plot displaying the relative contribution of continents and oceanic island groups to geographic connections in the combined *Platygaster*, *Synopeas*, *Telenomus* and *Trissolcus* BOLD BIN, GBIF and literature datasets. Numbers in parentheses indicate the total number of connections to that geographic grouping.

### GBIF data-mining and literature search

GBIF and literature searches returned 130 intercontinental taxa (37 Platygastriidae; 93 Scelionidae), with an overall discrepancy of plus 67 BINs (Table 3). The genera *Amblyaspis*, *Leptacis*, *Baeoneurella*, *Baryconus* and *Xenomerus* were present only in the BOLD BIN dataset (Table 3, Suppl. material 11). Conversely, the genera



**Figure 4.** Circularized p-distance neighbor-joining tree of the intercontinental and island BOLD BIN dataset. The BOLD BINs containing *Gryon aetherium*, *Platygaster sp.* (BOLD:ACI8542), *Psix striaticeps*, *Trissolcus basalis*, *Tr. japonicus* and *Telenomus sp.* (BOLD:ACY0393) are highlighted by an enlarged terminal cluster. Lines emanating from these clusters show generalized geographic localities where the BIN was detected. Solid black lines indicate different COI haplotypes. Solid red or blue lines indicate exact COI haplotype matches across continents. Striped red and blue lines indicate multiple exact COI haplotype matches found at that generalized geographic locality.

**Table 3.** Comparison of total platygastroid intercontinental and island distributions present in different data sources.

Taxon	GBIF/Literature	BOLD BINs	Discrepancy
Platygastriidae Total	37	72	+35 DNA
<i>Allotropa</i>	3	1	-2 DNA
<i>Amblyaspis</i>	0	5	+5 DNA
<i>Amitus</i>	4	1	-3 DNA
<i>Aphanomerus</i>	2	1	-1 DNA
<i>Euxestonotus</i>	1	2	+1 DNA
<i>Fidiobia</i>	2	2	-/-
<i>Inostemma</i>	1	0	-1 DNA
<i>Leptacis</i>	0	7	+7 DNA
<i>Metaclisis</i>	1	1	-/-
<i>Platygaster</i>	15	35	+20 DNA
<i>Synopeas</i>	7	13	+6 DNA
<i>Tetrabaeus</i>	1	0	-1 DNA
Scelionidae	93	129	+36 DNA
<i>Anteris</i>	1	1	-/-
<i>Aradophagus</i>	4	2	-2 DNA
<i>Baeoneurella</i>	0	1	+1 DNA
<i>Baeus</i>	2	4	+2 DNA
<i>Baryconus</i>	0	1	+1 DNA
<i>Calliscelio</i>	14	1	-13 DNA
<i>Dicroscelio</i>	1	2	+1 DNA
<i>Duta</i>	1	0	-1 DNA
<i>Dyscritobaeus</i>	3	1	-2 DNA
<i>Gryon</i>	4	6	+2 DNA
<i>Hadronotus</i>	4	5	+1 DNA
<i>Idris</i>	3	9	+6 DNA
<i>Platyscelio</i>	2	0	-2 DNA
<i>Probaryconus</i>	1	0	-1 DNA
<i>Psix</i>	3	1	-2 DNA
<i>Scelio</i>	1	0	-1 DNA
<i>Telenomus</i>	24	69	+45 DNA
<i>Trimorus</i>	1	7	+6 DNA
<i>Trissolcus</i>	24	11	-13 DNA
<i>Xenomerus</i>	0	1	+1 DNA

*Inostemma*, *Tetrabaeus*, *Duta*, *Platyscelio*, *Probaryconus* and *Scelio* were present only in the GBIF or literature datasets (Table 3, Suppl. material 6). In the process of analyzing the GBIF data, we encountered errors that were likely to be detected only by those with intimate knowledge of the group: *Trissolcus japonicus* (Ashmead) was listed as *Gryon japonicum* (Ashmead); records for *Trissolcus cultratus* (Mayr) were incomplete; *Calliscelio elegans* (Perkins) was listed as *Caenoteleia elegans* (Perkins); and *Telenomus dalmani* (Ratzeburg) was misspelled as *Telenomus dalmani*. Notable name changes are that *Trissolcus davatchii* (Javahery) is now treated as a junior synonym of *Tr. elasmuchae* (Watanabe) and *Tr. grandis* (Thomson) is a junior synonym of *Tr. belenus* (Walker).

## COI barcoding and identification of Platygastrids from the Jacksonville area

Talamas et al. (2017) provided names and images for species described from Jacksonville with primary types in the National Museum of Natural History. This enabled identifications for taxa that have yet to be revised with modern standards, at least for species with distinctive morphology. For example, *Synopeas cynipsiphilum* (BOLD record SUPER036-23) has a conspicuous divide between the mesoscutum and mesoscutellum that make it easy to recognize. For many others, diagnostic characters were either unknown or too subtle for us to confidently make a determination of species without studying the taxon in detail. In some cases, we did not make a determination because the specimens we sequenced were not the same sex as the primary type. As these taxa are revised, we are certain that more matches will be made between our vouchers and primary types from the region. Of the specimens that yielded COI barcode data, we matched seven specimens to primary types, totalling six species (Table 4).

Forty-nine specimens were COI barcoded from Timucuan Ecological and Historic Preserve and Buck Island, Jacksonville, Florida (Table 4). Twenty-eight specimens had BOLD identification hits greater than 97% matches (Table 4), representing 25 BINs. Specimen vouchers FSCA 00094179 and FSCA 00094185, both identified as *Telenomus* sp., were 100% matches to a specimen from San Diego, California, USA in the BIN BOLD:ACY0393 (Table 4). This is an intercontinental *Telenomus* BIN identified from the Insecta scripting procedure (Fig. 4), now with geographic data points in California, Florida and Bangladesh.

## COI barcoding of German specimens

Comparison of BOLD data to platygastrine specimens from the GBOL III barcoding initiative yielded 14 intercontinental BINs, representing 11 species in five genera (*Amblyaspis*, *Euxestonotus*, *Leptacis*, *Platygaster* and *Synopeas*). BOLD identified six species (seven BINs) only to family, two species (four BINs) to subfamily and three species (three BINs) with binomials. Of the three species identifications provided by BOLD, we verified two (*Pl. demades* and *Pl. sagana*) by comparison of voucher specimens to primary types, while one (*Pl. tuberosula*) was unverifiable. One more species (*E. error*) was unidentified in BOLD, but identifiable by our own examination. The remaining seven species were unidentifiable due to the superficial description impediment (Meier et al. 2022) in platygastrine taxonomy.

The distributions of two species (*Pl. demades* and putative *Pl. tuberosula*) can be explained by deliberate introductions for pest control on apple/pear and wheat, respectively. One species (*E. error*) is probably an unintentional introduction, moving with its host, the wheat midge *Sitodiplosis mosellana* (Géhin) (Echegaray et al. 2016). The biogeographic history of the remaining eight species could not be determined. It is possible that one of the unidentified *Platygaster* species (either BOLD:ACP1536 or BOLD:AAZ3286) is *Platygaster hiemalis* Forbes, deliberately introduced to New Zealand for control of the Hessian fly, *Mayetiola destructor* (Say) (Ferguson et al. 2007). However, the type material of *Pl. hiemalis* is unknown and the historical literature provides conflicting diagnoses.

## BOLD data-mining for Insecta and Araneae

The R script recovered 15,391 Insecta BOLD BINs with intercontinental and island distributions (Suppl. material 7). Members of 23 Insecta orders were present in the dataset, with only Mecoptera, Notoptera (Mantophasmatodea and Grylloblattodea) and Zoraptera absent. Due to conflicting taxonomies present in BOLD, the family and genus representation numbers are close estimates of higher-level diversity. Orders with the most family- and genus-group diversity were Coleoptera (66 families; 560 genera), Lepidoptera (77 families; 1,984 genera), Diptera (82 families; 899 genera), Hymenoptera (62 families; 736 genera) and Hemiptera (64 families; 454 genera) (Table 5). Lepidoptera and Diptera contained the most BINs in the dataset (Table 5). The R script recovered 499 Araneae BOLD BINs with intercontinental and island distributions (Suppl. material 18). Members of 42 Araneae families were present, comprising 224 genera (Suppl. material 18).

We randomly selected 769 BINs from the Insecta dataset for cursory validation of the scripting process (Suppl. material 2). Validated BINs were examined in Blattodea (1), Coleoptera (50), Diptera (166), Ephemeroptera (5), Hemiptera (38), Hymenoptera (96), Lepidoptera (391), Neuroptera (1), Orthoptera (6), Plecoptera (1), Psocodea (1), Thysanoptera (6) and Trichoptera (7). No validation BINs were discovered to be geographic false positives upon the initial pass. However, four BINs (BOLD:AAC6546, BOLD:AAP8198, BOLD:AAE7880 and BOLD:AAD4954) were recalculated in BOLD during the intervening time period of data gathering, analysis and validation. These four BINs had the appearance of being geographic false positives, but they were confirmed to be accurate by comparison to the records present in the analyzed data files. Major taxonomic conflicts were present in Blattodea (1/1; 100%), Coleoptera (12/50; 24%), Diptera (44/166; 26%), Hemiptera (15/38; 39%), Hymenoptera (31/96; 32%), Lepidoptera (97/391; 25%), Neuroptera (1/1; 100%), Orthoptera (3/6; 50%) and Trichoptera (4/7; 57%) (Suppl. material 2). There were far fewer minor taxonomic conflicts (Lepidoptera = 19, Hymenoptera = 3, Hemiptera = 1, Diptera = 4) (Suppl. material 2). Twenty-five BINs from the Araneae dataset were randomly selected for validation of the scripting process (Suppl. material 19). Validated BINs were examined in Araneidae (1), Clubionidae (1), Gnaphosidae (1), Linyphiidae (10), Lycosidae (4), Pholcidae (1), Tetragnathidae (1), Theridiidae (4) and Thomisidae (2) (Suppl. material 19). No validation BINs were discovered to be geographic false positives upon the initial pass. Major taxonomic conflicts were present in Araneidae (1/1; 100%), Clubionidae (1/1; 100%), Linyphiidae (3/10; 30%), Lycosidae (1/4; 25%) and Thomisidae (1/2; 50%) (Suppl. material 19).

Greater than 80% of recovered Insecta BINs were present on two continents or islands (Fig. 5, Suppl. material 8), totalling 254 unique combinations of continent/island points (Suppl. material 9). Europe–North America (4,207 points), Asia–Europe (2,324 points), North America–South America (2,248 points), Asia–Australia (827 points) and Africa–Asia (665 points) were the five most common geographic combinations captured within Insecta BINs (Suppl. material 9). In total, North America (12,612 connections), Europe (11,402 connections) and Asia (8,728 connections)

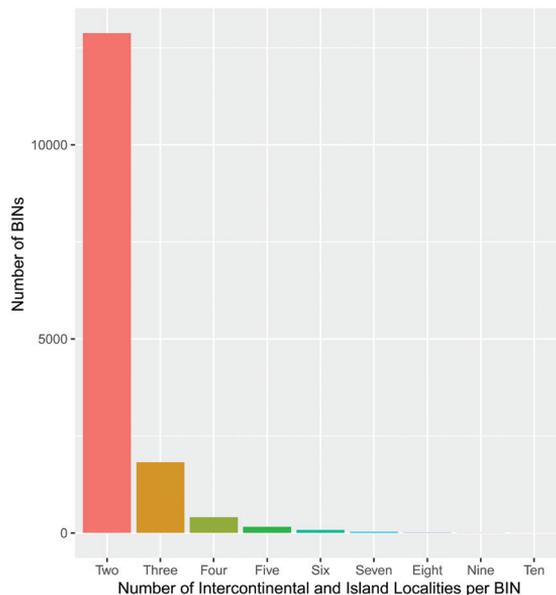
**Table 4.** BOLD identification engine identifications for COI barcoded platygastroid specimens collected at Timucuan Ecological and Historic Preserve and Buck Island (Jacksonville Port Authority).

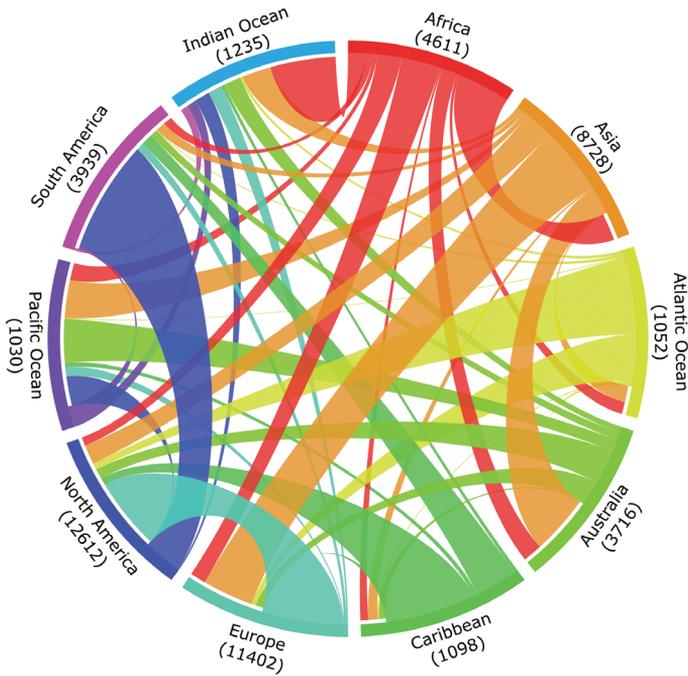
Processid	Sampleid	Morphological Identification	Topotype	% Match	Match Lowest Taxonomy	Match BIN
SUPER027-23	FSCA 00094159	<i>Baeus</i>	No	97.19	Scelionidae	BOLD:ACZ5774
SUPER008-23	FSCA 00094151	<i>Baryconus floridanus</i>	Yes	92.4	Scelionidae	BOLD:ABA5967
SUPER034-23	FSCA 00094155	<i>Calotelea</i>	No	100	Scelionidae	BOLD:AAN8024
SUPER043-23	FSCA 00093888	<i>Calotelea bicolor</i>	No	93.78	Scelionidae	BOLD:ADY8276
SUPER047-23	FSCA 00091193	<i>Hadronotus bicolor</i>	No	99.36	<i>Gryon bicolor</i>	BOLD:AAN8046
SUPER048-23	FSCA 00090993	<i>Hadronotus bicolor</i>	No	98.84	<i>Gryon bicolor</i>	BOLD:AAN8046
SUPER049-23	FSCA 00091003	<i>Hadronotus bicolor</i>	No	99.36	<i>Gryon bicolor</i>	BOLD:AAN8046
SUPER002-23	FSCA 00090995	<i>Hadronotus carinatifrons</i>	No	99.5	<i>Hadronotus carinatifrons</i>	BOLD:AET1244
SUPER042-23	FSCA 00093865	<i>Hadronotus chelinideae</i>	No	98.87	<i>Gryon chelinideae</i>	BOLD:ACN3082
SUPER040-23	FSCA 00097242	<i>Inostemma</i>	No	98.79	Platygastriidae	BOLD:ACT8287
SUPER045-23	FSCA 00093946	<i>Leptacis longipes</i>	Yes	93.63	<i>Leptacis</i>	BOLD:ABV2678
SUPER044-23	FSCA 00093928	<i>Leptacis puncticeps</i>	Yes	97.47	Platygastriidae	BOLD:AEE0956
SUPER001-23	FSCA 00091067	<i>Metaclisis</i>	No	92.26	Platygastriidae	BOLD:AEC9177
SUPER037-23	FSCA 00097245	<i>Metanopedias brunneipes</i>	Yes	98.77	Platygastriidae	BOLD:ABY3815
SUPER028-23	FSCA 00094161	<i>Phanuromyia</i>	No	91.2	Scelionidae	BOLD:ACJ7306
SUPER033-23	FSCA 00094146	<i>Phanuromyia</i>	No	99.84	<i>Telenomus autumnalis</i> (unavailable name)	BOLD:ACM1917
SUPER004-23	FSCA 00094172	<i>Phanuromyia</i>	No	89.62	Scelionidae	BOLD:AEN8490
SUPER005-23	FSCA 00094176	<i>Phanuromyia</i>	No	86.37	Scelionidae	BOLD:ADH6867
SUPER006-23	FSCA 00094152	<i>Phanuromyia</i>	No	86.9	Scelionidae	BOLD:ADH6867
SUPER007-23	FSCA 00094154	<i>Phanuromyia</i>	No	98.72	<i>Telenomus</i>	BOLD:AAN8100
SUPER022-23	FSCA 00095852	<i>Platygaster</i>	No	96.54	Platygastriidae	BOLD:AAN8090
SUPER039-23	FSCA 00097243	<i>Platygaster</i>	No	87.64	Platygastriidae	BOLD:ADI9080
SUPER010-23	FSCA 00094167	<i>Scelio floridanus</i>	Yes	98.74	Scelionidae	BOLD:ACA7140

Processid	Sampleid	Morphological Identification	Topotype	% Match	Match Lowest Taxonomy	Match BIN
SUPER029-23	FSCA 00094138	<i>Scelio floridanus</i>	Yes	98.89	Scelionidae	BOLD:AEY5850
SUPER009-23	FSCA 00094175	<i>Scelio incertus</i>	No	99.53	Scelionidae	BOLD:ACA7140
SUPER041-23	FSCA 00093881	<i>Scelio opacus</i>	No	95.27	Scelionidae	BOLD:ACT6721
SUPER035-23	FSCA 00093874	<i>Scelio pumilis</i>	No	99.81	<i>Scelio</i>	BOLD:ACA7141
SUPER003-23	FSCA 00095771	<i>Synopeas</i>	No	93.94	Platygastriidae	BOLD:ADH8704
SUPER038-23	FSCA 00095854	<i>Synopeas</i>	No	99.53	Platygastriidae	BOLD:AAV6787
SUPER017-23	FSCA 00094197	<i>Synopeas</i>	No	93.38	Platygastriidae	BOLD:ACM5719
SUPER018-23	FSCA 00095010	<i>Synopeas</i>	No	94.16	Platygastriidae	BOLD:ADH9879
SUPER019-23	FSCA 00095011	<i>Synopeas</i>	No	93.62	Platygastriidae	BOLD:ADH9879
SUPER020-23	FSCA 00095851	<i>Synopeas</i>	No	99.84	<i>Synopeas</i>	BOLD:AEP1939
SUPER021-23	FSCA 00097239	<i>Synopeas</i>	No	99.68	Platygastriidae	BOLD:AEP1939
SUPER036-23	FSCA 00097487	<i>Synopeas cynipsiphilum</i>	Yes	99.04	<i>Synopeas</i>	BOLD:ADX3415
SUPER011-23	FSCA 00094179	<i>Telenomus</i>	No	100	Scelionidae	BOLD:ACY0393
SUPER012-23	FSCA 00094185	<i>Telenomus</i>	No	100	Scelionidae	BOLD:ACY0393
SUPER013-23	FSCA 00094149	<i>Telenomus</i>	No	100	Scelionidae	BOLD:ACV4748
SUPER014-23	FSCA 00094165	<i>Telenomus</i>	No	100	Scelionidae	BOLD:AEO7335
SUPER015-23	FSCA 00094156	<i>Telenomus</i>	No	100	<i>Telenomus</i>	BOLD:AAN8031
SUPER016-23	FSCA 00094137	<i>Telenomus</i>	No	100	<i>Telenomus</i>	BOLD:ABW3189
SUPER032-23	FSCA 00094153	<i>Telenomus</i>	No	100	Scelionidae	BOLD:ACI3554
SUPER046-23	FSCA 00093915	<i>Telenomus</i>	No	99.35	<i>Telenomus</i> sp. SL017	BOLD:AEI6588
SUPER023-23	FSCA 00094140	<i>Telenomus</i>	No	97.11	Scelionidae	BOLD:ACX8754
SUPER024-23	FSCA 00094150	<i>Telenomus</i>	No	93.81	Scelionidae	BOLD:ADY7126
SUPER025-23	FSCA 00094139	<i>Telenomus</i>	No	99.68	<i>Telenomus</i>	BOLD:ABY2759
SUPER026-23	FSCA 00094173	<i>Telenomus</i>	No	93.84	<i>Telenomus podisi</i>	BOLD:ADK2938
SUPER030-23	FSCA 00094249	<i>Trimorus</i>	No	94.94	Scelionidae	BOLD:AEJ7657
SUPER031-23	FSCA 00094136	<i>Trimorus</i>	No	99.52	<i>Trimorus</i>	BOLD:ABV9390

**Table 5.** Taxonomic summary of Insecta BINs displaying geographic distributions spanning continents and islands.

Order	Families	Genera	BINs
Archaeognatha	1	1	1
Zygentoma	1	3	3
Odonata	8	51	91
Ephemeroptera	7	18	37
Dermaptera	4	7	11
Plecoptera	4	8	10
Orthoptera	6	44	62
Embioptera	2	3	5
Phasmatodea	2	4	4
Mantodea	1	5	7
Blattodea	7	31	60
Psocodea	21	30	107
Thysanoptera	3	29	86
Hemiptera	64	454	924
Hymenoptera	62	736	3,056
Raphidioptera	1	1	1
Neuroptera	5	15	43
Strepsiptera	2	2	2
Coleoptera	66	560	1,121
Trichoptera	17	47	126
Lepidoptera	77	1,984	5,336
Siphonaptera	3	6	12
Diptera	82	899	4,286
<b>Total: 23</b>	<b>446</b>	<b>4,938</b>	<b>15,391</b>

**Figure 5.** Histogram displaying the percentage of Insecta BINs with a given number of continent and island data points.



**Figure 6.** Circos plot displaying the relative contribution of continents and oceanic island groups to geographic connections in the Insecta BOLD BIN dataset. The single data point for Antarctica was eliminated from this visualization. Numbers in parentheses indicate the total number of connections to that geographic grouping.

were the most connected geographic categories (Fig. 6, Suppl. material 10). Europe–North America (5,563 connections), Europe–Asia (3,549 connections), North America–South America (2,718 connections), North America–Asia (1,520 connections), Africa–Asia (1,458 connections) and Australia–Asia (1,345 connections) were the most common connections in Insecta BINs (Fig. 6, Suppl. material 10).

## Discussion

### Lessons from Platygastroidea

Our BOLD data-mining approach confirmed several well-characterized cases of parasitoid range expansion in *Tr. basalis*, *Tr. japonicus*, *Tr. hyalinipennis*, *Te. remus*, *G. aetherium*, *Ps. striaticeps* and *Pl. demades*. Therefore, the geographic distribution patterns found in the platygastroid dataset are generally considered credible even when lacking any directionality. The effort of COI barcoding topotypical specimens can be helpful for determining directionality, particularly if the specimens match primary types described a long time ago. Antiquity of specimens does not preclude the possibility that they were adventive at the time of collection, but it gives, at minimum, a historical perspective.

Exact COI barcode matches from geographically disparate populations might be the most conclusive evidence of a new adventive population within our framework. However, even among the validated cases mentioned above, only *Tè. remus*, *G. aetherium* and *Ps. striaticeps* were scored as exact long-distance matches. *Trissolcus japonicus* and *Tr. basalis* both have the appearance of exact intercontinental matches, but were scored inconclusive due to sequence length variation. *Trissolcus hyalinipennis* and *Pl. demades* have no evidence for exact intercontinental matches in our dataset. Specimens identified as *Pl. demades* are divided into five BOLD BINS, two of which contain populations introduced into Canada and the USA (Mason et al. 2017). These Nearctic *Pl. demades* BINS were not captured by our method and show that the expectation of exact matches may be unnecessary when inferring range expansion, although it does lend additional confidence. The BIN calculations still captured relevant information in these cases even though additional geographic data points were present in neighboring BINS.

A majority of the intercontinental platygastroid BINS were unidentified below the family level at the beginning of this study, affording our group a “clean” taxonomic slate on which to analyze and interpret these results. Alternatively, the identifications for many of the validated intercontinental Insecta BINS were conflicted, which confounds interpretation of the data. Conflicting taxonomies present in BOLD BINS complicated our ability to extract and summarize higher-level taxonomic data from the Insecta dataset. Even if these taxonomic conflicts can be rectified or understood on a case-by-case basis by expert systematists, that database problem is likely to persist in future analyses. We advocate that experts use our baseline dataset to closely examine the intercontinental BINS in their group of interest and make informed judgements about their veracity.

Many of the BOLD BIN criticisms levelled by Meier et al. (2022) are applicable to our approach. We found that BOLD BIN recalculations affected our study, once again demonstrating their instability. A few of our validation BINS were missing when they were queried back to BOLD for checking, having been recalculated between the time of database mining and data analysis. This feature of BOLD BINS will certainly complicate repetition of our analyses in the future. For better or worse, we must rely on BINS calculated database-wide for our proposed method because they are tied to the specimen metadata in an efficient way for mining. To further verify our relatively small (nearly 10,000 COI barcodes) platygastroid dataset, the sequences could be aligned, phylogenetically analyzed and those results used to provide *de novo* mOTUs by varying methods with more public documentation than RESL (Ratnasingham and Hebert 2013). However, this smaller sampling of barcodes would influence mOTU calculations. The most rigorous results would be obtained by analyzing all available Platygastroidea data in BOLD, comprising well over 140,000 COI barcodes and growing. Such large analyses would firmly move our method into the realm of bioinformaticians and supercomputing, rather than being repeatable by scientists with less capability for big data. We consider it a major strength that our approach can be performed on an internet-connected, standard desktop computer with freely-available statistical software.

Our case study of Platygastriinae using GBOL specimens indicated that BINs overestimated “species richness” and we reiterate that the BINs in the Insecta and Platygastroidea dataset do not necessarily equate to species. Results for poorly-known or hyperdiverse insect groups must be interpreted with caution.

### Natural or adventive distributions in Platygastroidea?

Platygastroid wasps are one of the most dominant flying insect groups worldwide, with a high rate of geographically structured community turnover and high taxonomic neglect, further complicating faunistic studies (Srivathsan et al. 2023). Srivathsan et al. (2023) discovered 44 platygastroid mOTUs in multiple sampling sites. They also reported that usually less than 3% (1–9% within a given insect family) of their mOTUs in the top 20 most dominant insect families were found at multiple sites. About five members of our platygastroid BIN dataset display geographic ranges which may be “false positives” based on our intentions. For example, BINs shared between the Republic of Georgia and Iran or Egypt and Saudi Arabia were scored as intercontinental even though these countries are in close geographic proximity. Conservatively, we detected 195 platygastroid BINs comprising specimens with vast geographic ranges. As of February 2023, BOLD analytics has calculated 11,468 Platygastroidea BINs. From our study, approximately 1.7% of available platygastroid BINs display this striking distribution pattern.

Determining whether these distributions are natural or adventive for most of the platygastroid BINs is difficult pending taxonomic revisions and follow-up research. Gilligan et al. (2020) suggested that a species association with Beringia supports hypothetically natural Holarctic distributions in Tortricidae (Lepidoptera), while also providing a good list of expectations for Holarctic species versus recent introductions. For truly Holarctic organisms, expectations include: 1) no direct evidence of introduction, 2) association with native hosts, 3) initial reports from inland areas, 4) lack of recent range expansion and 5) presence in the Arctic biogeographic region (Gilligan et al. 2020). These expectations are a useful framework for assessing many of the intercontinental platygastroid BINs present in Canada, Europe and northeast Asia.

Many Platygastroidea distributions in the BOLD dataset were from the Southern Hemisphere, between the Northern and Southern Tropics or island localities, precluding the need to consider a naturally Holarctic distribution as an explanation for the pattern. We think those cases are best considered introductions mediated by human activity. However, in some *Platygaster* and *Telenomus* BINs, the locality data imply enormous geographic ranges across the entirety of Canada, northern and central Europe, northeastern Asia and other spurious localities. For example, *Telenomus* sp. (BOLD:AAV1142; 461 public barcodes) has occurrences across Canada including Nunavut in the north, south to desert regions of eastern California and eastern Europe. One unidentified platygastriine (BOLD:ABW3192; 37 public barcodes with several exact matches) has occurrences in eastern and western Canada, Germany, the Russian Far East, South Africa and California. These geographic ranges encompass several

climates and biomes, showing the apparent ability of the wasps to tolerate dramatically different environmental conditions for at least a short period, considering that species that fail to establish would have to persist long enough to be collected.

Historically, the Palearctic and Nearctic Regions have been regarded as separate by platygastroid taxonomists. Early European authors rarely made comparisons to the fauna of neighboring countries, let alone distant continents. Likewise, early American hymenopterists often treated the Nearctic fauna as unique. This approach contrasts with that of early American lepidopterists, who tended to misidentify Nearctic species as similar-looking European species (Gilligan et al. 2020). The discrepancy may be explained by the availability of detailed keys and illustrations for Lepidoptera in the 19<sup>th</sup> century, while descriptions of Platygastroidea were generally short and vague.

Contrary to the assumptions of the past, the results of our study suggest that some platygastroid genera, such as *Platygaster*, include many naturally Holarctic species as well as human-mediated introductions. Landry et al. (2013) came to a similar conclusion for Lepidoptera, with the added benefit of host plant data to help distinguish natural from anthropogenic distributions. It is difficult to make such determinations for Platygastriinae, as little is known of their host repertoire and Cecidomyiidae is itself a dark taxon. However, our results also indicate a high number of intercontinental cecidomyiid BINs, which matches well with the parasitoid distributions.

Constraints due to a lack of data are a consistent theme throughout platygastroid taxonomy, especially when compared with better-studied groups of insects. Our method offers a path to gather and interpret the available data, albeit with limitations. For example, the Nearctic and western Palearctic were remarkably well sampled, allowing for more detailed examination of distribution patterns. On the other hand, the Pacific Islands and Caribbean yielded no *Platygaster* or *Synopeas* records. This likely reflects reality in the Pacific Islands but is a result of under sampling in the Caribbean, a distinction which cannot be made by our method alone. Ashmead (1900) recorded 29 species of platygastriine wasps from St. Vincent and Grenada, while the only platygastriine in Hawaii is considered an accidental introduction (Drake 1969).

## Digital morphology in BOLD

Specimen images in BOLD allowed us to provide a list of genus level identifications for about one fifth of all platygastroid BOLD BINs. This has just begun the process of overcoming taxonomic impediments in the group and the database more broadly, as many thousands of BINs remain to be examined and identified. Platygastroid wasps are generally small insects (0.5 to 10 mm), making species level characters difficult to assess without proper microscopy and high-resolution images. Regardless, the image quality and habitus views in BOLD were generally sufficient to identify BINs to genus. We encourage systematists to examine BIN images in their group of expertise to see if this process can be repeated for other under-studied insect families. Given that we discovered several BINs which contained genera lacking any other DNA sequence data, it is likely that more such cases remain to be found.

Data presented here suggest that taxonomic revisions of platygastroids should be targeted at the global level as much as possible, even if there are feasibility concerns. A consideration of data from across the world will likely be necessary for the accurate and precise description of some sections of platygastrid and scelionid biodiversity. The BIN database should be preemptively searched to quickly quantify diversity present in an area and inform the taxonomic approach. Our genus level identification of BINS has already facilitated one such study. Melotto et al. (2023) described a new species, *Synopeas maximum* Awad & Talamas, which parasitizes an emerging pest of soybean. Given that soybean is a cosmopolitan crop, it was a possibility that this parasitoid was adventive. A phylogenetic analysis of *Synopeas* BINs enabled them to determine that the closest relatives of *S. maximum* are from North America, suggesting that it was not adventive. Furthermore, specimen images in BOLD allowed them to assess if certain morphological characters corresponded to a monophyletic species group.

On another research track, the molecular evolution of COI barcodes across Animalia was evaluated by Pentisaari et al. (2016), demonstrating that parasitic groups had many convergent amino acid variations. Several COI barcode amino acid phenotypes have been noted in Platygastroidea and some appear informative at the genus level (Talamas et al. 2021). Increased BOLD resolution for Platygastroidea genera could allow for new analyses of these patterns for the superfamily (Chen et al. 2021).

## Implications for biological control programs

These analyses highlight the urgent need for more detailed and comprehensive approaches (Talamas et al. 2021) when identifying biological control agents, whether classical or augmentative. This is especially the case for platygastroid egg parasitoids; they are small, cryptic and their biodiversity dwarfs what has been adequately described. They also attack small, cryptic and poorly-described insect life stages. COI barcoding of proposed classical biological control agents must occur immediately at program outset. New data can then be quickly assessed as distinct or not present in BINs calculated from other non-native areas. Putatively “native” communities of parasitoids being evaluated for use as augmentative biological control agents should be similarly treated. Our results and those of Srivathsan et al. (2023) demonstrate the significant likelihood of unintended and undocumented platygastroid introductions.

## Insecta and Araneae

Approximately 84% (12,876/15,391) of the recovered intercontinental and island Insecta BINs were detected across two geographic categories. When these geographic categories are contiguous or adjacent, they are the most likely to be ‘false positives’ based on our data-mining approach. For example, BINs present in lowland rainforest habitats of both Costa Rica and Colombia were scored as intercontinental even though these are probably natural distributions (North America/South America; 2,248 BINs). Indeed, there are many such cases in the dataset, especially among Neotropical

Lepidoptera BINs. In the Palearctic, Europe and Asia (2,324 BINs) were connected at a similar magnitude. BINs detected across contiguous landmasses warrant additional scrutiny by taxonomic experts to determine their status as adventive or natural distributions. Just over 2,500 Insecta BINs were detected from three or more continents or island chains. This set of BINs contains insect species which appear to be truly successful global invaders. Our results were intuitive for insect BOLD BINs with the most widespread geographic data; these included species long associated with human activity. For example, *Ctenocephalides felis* (cat flea; BOLD:AAY6332), *Aedes aegypti* (yellow fever mosquito; BOLD:AEI9358), *Culex quinquefasciatus* (southern house mosquito; BOLD:AAA4751) and *Aphis gossypii* (cotton aphid; BOLD:AAA3070) were recovered in the analysis with 9 or 10 intercontinental and island data points.

The highly structured global sampling of flying insect communities undertaken by Srivathsan et al. (2023) provides some meaningful context for our results. In that study, greater than 97% of mOTUs from the most dominant flying insect families were sampled only at a single site, suggesting massive community turnover between sites (Srivathsan et al. 2023). Our BIN dataset, which notably was not designed for statistical testing a priori nor sorted by biological traits, suggests that about 3.15% of the BOLD Insecta BINs are distributed across continents and islands (489,156 Insecta BINs as of September 2023). Simpson et al. (2021) considered 3,747 insects and 58 spiders to be adventive in the continental United States. That dataset serves as a valuable baseline of verified introductions for comparison to the BOLD BINs provided herein. We found 12,612 intercontinental Insecta BINs with a distribution that included North America, indicating a large discrepancy between these methods. Our larger number may be an artifact of the data-mining approach, as mentioned previously, but may also reflect undetected introductions. Rectifying these datasets is a herculean task, given their size and the taxonomic expertise needed. However, given the magnitude of the agricultural, environmental and economic consequences of invasive species, this should be a research priority for biosecurity experts, bioinformaticians and systematists.

## Regulatory and biosecurity applications

Biosecurity practices entail anticipatory risk assessment and preparedness, methods of surveillance, emergency management and policy enforcement (Barker and Francis 2021). This dataset has potential applications for risk assessment and surveillance. Our approach sought to use large geographic spans in BINs with the goal of identifying putatively adventive platygastroids. Small adjustments to the data-mining and analysis methods could be made to focus on administrative borders instead. BOLD data have already been used to inventory fauna at the country level (Geiger et al. 2016; Hebert et al. 2016), making new BIN additions to Canada and Germany straightforward to detect.

Emerging infectious disease surveillance programs have implemented several automated, internet-based data-mining approaches for intelligence gathering (Stevens 2021). Similarly, automation and scheduled repetition of a modified version of our analysis could provide information supporting risk assessment and invasion prepared-

ness as BINs accumulate in new geographic areas. Detection at the earliest stages of invasion, followed by rapid response, provides the best economic and environmental outcomes. This proposed method of biosecurity surveillance will probably not be timely enough to meet early detection criteria; once new data points are detected in an area, the proverbial cat is already out of the bag. Agencies also rely on in-hand specimens for diagnosis of regulated species and are unlikely to take a decentralized BIN record as definitive evidence of new introductions. However, this may be a situation analogous to several cases of verified arthropod introductions that were first noticed or tracked as records in iNaturalist and BugGuide (Iwane 2018; Halbert et al. 2020; USDA 2021; Hayden et al. 2022; Chuang et al. 2023; iNaturalist 2023; VanDyk 2023).

Further scrutinizing the Insecta BINs with consideration for specimen spatiotemporal data might also inform pathway analyses for species moving via global trade, weather phenomena or animal migration events. Data from the Jacksonville area uncovered an intercontinental *Telenomus* species, with matching specimens collected in 2014 and 2015 from coastal areas of Bangladesh and California. Relevant agencies could have been alerted to the risk of this new introduction, possibly centered around international seaports-of-entry, eight years prior if the available data were scanned systematically. Thus, nearly a decade of potential research progress on this unidentified, globally-mobile *Telenomus* went unrealized. Cases like these might be valuable input for pathway models or agent-based models of long-distance insect dispersal. Detailed examination of the Insecta BIN dataset should reveal similar cases for groups with a widely-variable range of life history traits, dispersal potentials and introduction histories or modalities.

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## Supplementary material I

### Country to continent and island group assignments

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee  
 Data type: xlsx

Explanation note: File detailing country and island continent assignments.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl1>

## Supplementary material 2

### **Randomized BOLD BINs for validation of the Insecta dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Table detailing which Insecta BINs were validated by manual examination.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl2>

## Supplementary material 3

### **Intercontinental and island Platyastroidea COI dataset alignment**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: fas

Explanation note: DNA alignment of COI barcodes from intercontinental and island platygastroids.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl3>

## Supplementary material 4

### **Platygastroidea COI dataset NJ tree.tre**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: tre

Explanation note: P-distance NJ tree of platygastroid COI barcodes.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl4>

## Supplementary material 5

### **Platygastroidea COI dataset NJ tree annotated FIGTREE**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: tre

Explanation note: FigTree file of annotated p-distance NJ tree Newick tree.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl5>

## Supplementary material 6

### **Intercontinental and island records for targeted Platygastroidea in GBIF and the literature**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Data summary for platygastroid taxa with GBIF and literature Records.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl6>

## Supplementary material 7

### **List of intercontinental and island Insecta BINs with identification metadata**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: List of Insecta BINs recovered by the scripting procedure as intercontinental or island hits, BINs listed under Identification Remarks.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl7>

## Supplementary material 8

### **Number of distinct continent or island groupings recovered per Insecta BIN**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: csv

Explanation note: Table displaying number of continent and island hits per Insecta BIN.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl8>

## Supplementary material 9

### **Unique continent and island hit combinations in Insecta dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: csv

Explanation note: Table showing frequencies of geographic combinations in the Insecta BIN dataset.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl9>

## Supplementary material 10

### **Pairwise geographic hit comparisons for the Insecta BIN dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Pairwise matrix used to generate Circos plot in Figure 1.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl10>

## Supplementary material 11

### **Summary of intercontinental and island Platyastroidea BINs**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl11>

## Supplementary material 12

### **Pairwise geographic hit comparisons for the Platyastroidea BIN, GBIF, and literature dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Matrix used to generate Circos plot in Figure 4.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl12>

### **Supplementary material 13**

#### **Pairwise geographic hit comparisons for the Platygaster BIN, GBIF, and literature dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Matrix used to generate Circos plot in Figure 5.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl13>

### **Supplementary material 14**

#### **Pairwise geographic hit comparisons for the Synopeas BIN, GBIF, and literature dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Matrix used to generate Circos plot in Figure 5.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl14>

## Supplementary material 15

### **Pairwise geographic hit comparisons for the *Telenomus* BIN, GBIF, and literature dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Matrix used to generate Circos plot in Figure 5.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl15>

## Supplementary material 16

### **Pairwise geographic hit comparisons for the *Trissolcus* BIN, GBIF, and literature dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Matrix used to generate Circos plot in Figure 5.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl16>

## Supplementary material 17

### **Platygastroidea BIN identifications using digital morphology infrastructure**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Table of wasp identifications provided to BOLD by using existing digital morphology infrastructure.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl17>

## Supplementary material 18

### **List of intercontinental and island Araneae BINs with identification metadata**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: csv

Explanation note: List of BOLD metadata for intercontinental and island Araneae identified using the scripting procedure. BINs are under Identification Remarks.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl18>

## Supplementary material 19

### **Randomized BOLD BINs for validation of the Araneae dataset**

Authors: Matthew R. Moore, Elijah J. Talamas, Jonathan S. Bremer, Natalie McGathey, James C. Fulton, Zachary Lahey, Jessica Awad, Cheryl G. Roberts, Lynn A. Combee

Data type: xlsx

Explanation note: Table of Araneae BINs manually examined for validation of Araneae BOLD scripting procedure.

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Link: <https://doi.org/10.3897/neobiota.88.106326.suppl19>

# Disruptions caused by invasive species and climate change on the functional diversity of a fish community

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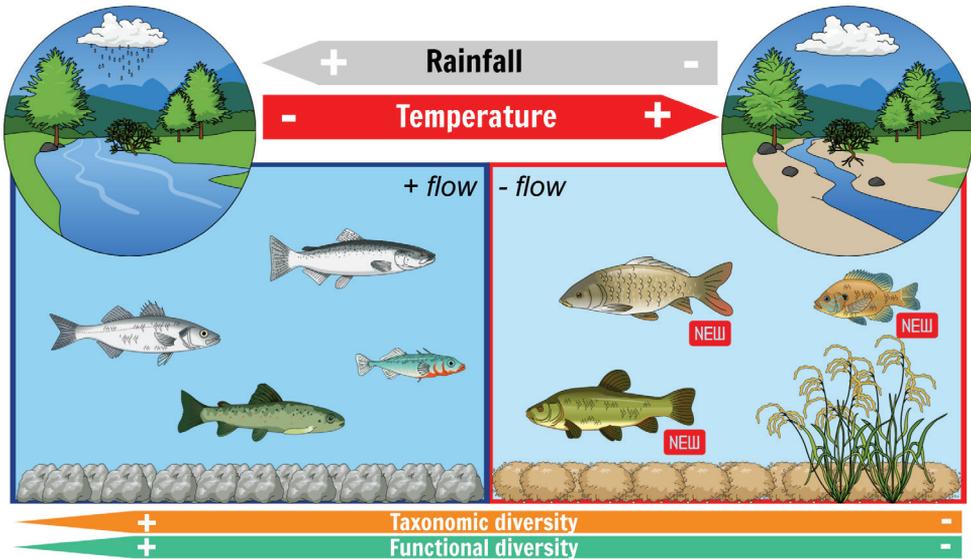
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## Abstract

As the effects of climate change continue to intensify, non-native species are becoming more prevalent in estuarine ecosystems. This has implications for the taxonomic and functional diversity of fish communities. Historically, biodiversity has been a synonym of taxonomic diversity, however this approach often fails to provide accurate insights on ecosystem functioning and resilience. To better understand how climate change is impacting fishes and their traits' composition, a long-term dataset from Minho Estuary (NW Iberian Peninsula) fish assemblage was analyzed. The results suggest that climate change and extreme weather events altered the prevailing trait modalities of fishes, which led to the overall decrease in functional diversity of the fish assemblage over the course of a decade. This decrease is associated to the loss of some trait modalities that are exclusively found in native species. On the other hand, the invasive species added novel traits associated with the conditions of high temperatures and low precipitation regime currently observed in the studied area. Our results highlight that the shift in the presence and dominance of some traits is directly influenced by climatic changes. Also, despite the addition of novel modalities by the invasive species, the fish assemblage is now less functional and taxonomically diverse than previously.

## Graphical abstract



## Keywords

biodiversity, biological invasions, climatic events, ecosystem functioning, native species, traits

## Introduction

Climate change is one of the biggest threats to biodiversity currently (IPBES 2019; Reid et al. 2019), and nowadays many taxonomic groups and ecosystems around the world have already been affected (Alan Pounds et al. 2006; Moritz et al. 2008; Jarić et al. 2019). As global temperatures rise and weather patterns shift, ecosystems across the globe are undergoing drastic changes in their stability and functioning (Markham 1996; Walther et al. 2002; Parmesan and Yohe 2003). This can have a significant impact on the functional diversity of an ecosystem, as climate change can either favor certain species or cause the local extinction of others (Thuiller et al. 2006). The effects of climate change on functional diversity vary depending on the types of species present in a particular ecosystem. For example, species that are heat-tolerant may spread and flourish during periods of warmer climate; this is expected, for example, for the largemouth bass (*Micropterus salmoides*) in Iberian Peninsula (Bae et al. 2018). Other species, however, may be negatively impacted by shifts in temperature, which may translate into changes in species physiology, phenology, behavior, and geographic range (Robinson et al. 2009; Sorte et al. 2010; Hauser et al. 2018; Howard et al. 2020); this is the case for several tropical and subtropical fish species that have reach the northern Gulf of Mexico (Fodrie et al. 2010). Additionally, some species may require certain

conditions for foraging or nesting, and climate change may limit their access to these resources, thus preventing them from obtaining enough food and reproducing normally (Segev et al. 2014; Descamps et al. 2017). These factors collectively contribute to changes in functional diversity within ecosystems, leading to disruptions in populations and communities that vary depending on the compatibility between a species' ecological traits and the prevailing climatic conditions.

Historically, biodiversity has been associated to taxonomic diversity (Cardoso et al. 2014). However, this approach many times failed to provide insights into ecosystem functioning. Therefore, the use of traits and functional diversity indices alongside with taxonomic diversity provide a more holistic understanding about biodiversity (Hulme and Bernard-Verdier 2018). Both types of diversity metrics are important when evaluating the biodiversity of a given ecosystem (Villéger et al. 2010; Moore 2013; Teittinen and Virta 2021). In fact, due to the importance of functional diversity to biodiversity assessments, the number of scientific manuscripts integrating functional diversity into the ecological assessments has been increasing exponentially in recent years (Palacio et al. 2022). In general, ecosystems with high levels of both taxonomic and functional diversity are more stable and resilient to disturbances than ecosystems with low levels of diversity (Walker et al. 1999; Cadotte et al. 2011). By looking at the range of functions that different species perform in the ecosystem, the functional diversity indices provide a more in depth assessment of the ecosystem's overall condition. This is because a greater variety of functions creates greater redundancy within the system, meaning that if one species is lost, there are others that can perform its role in the ecosystem (Biggs et al. 2020).

On the other hand, climate change plays an important role in the establishment and spread of invasive species, a phenomenon that is widely recognized (Stachowicz et al. 2002). Currently, biological invasions are one of the most important topics in ecology (Anderson et al. 2021). The impacts of biological invasions can be extensive and often detrimental to native ecosystems (Pyšek et al. 2020). Invasive species can disrupt food webs (Wainright et al. 2021), alter habitats (Crooks 2002; Guy-Haim et al. 2018), displace native species (Catford et al. 2018), cause biodiversity loss (Pyšek et al. 2020), alter ecosystem functioning (Haubrock et al. 2021), and lead to significant social and economic impacts (Simberloff et al. 2013; Diagne et al. 2020). Invasive species can also out-compete native species for resources (Catford et al. 2018; Ferreira-Rodríguez et al. 2018), leading to a decline in diversity (Mollot et al. 2017; Williams-Subiza and Epele 2021) that can affect the entire ecosystem and affect various taxonomic groups that are directly or indirectly linked to them (Crooks 2002; Guy-Haim et al. 2018; Goedknecht et al. 2020; Vivó-Pons et al. 2020). Additionally, climate change is likely to exacerbate the impacts of biological invasions (Rahel and Olden 2008; Diez et al. 2012; Bellard et al. 2013), as rising temperatures and changes in precipitation regimes create new opportunities for the establishment of non-native species in new areas (Stachowicz et al. 2002; McKnight et al. 2021; Souza et al. 2022b).

While it is well documented that biological invasions usually have a negative impact on taxonomic diversity (Pyšek et al. 2020; Ilarri et al. 2022), few studies have

addressed the effects of this phenomenon on functional diversity (but see Sîrbu et al. 2022; Renault et al. 2022). Invasive species can fill empty trait gaps in the invaded ecosystems or replace the ones occupied by native species (Loiola et al. 2018), thereby disrupting functional diversity in these ecosystems (Hatfield et al. 2022; Linares et al. 2022). While the decline in taxonomic diversity can be accompanied by the loss of certain traits, it is crucial to understand how the decline in taxonomic diversity translates into changes in functional diversity, particularly in the context of simultaneous biological invasions and climate change. Hence, it is imperative to monitor ecosystem function and functional diversity as a means to address the threats posed by biological invasions and climate change. Through a continuous tracking of these indices, ecologists can identify areas at risk of species or functional loss (Santini et al. 2017) and pinpoint regions where appropriate management or conservation efforts are required.

Among the species groups particularly vulnerable to climate change and biological invasions are estuarine fishes (Gillanders et al. 2011; Souza et al. 2018; Lauchlan and Nagelkerken 2020; Ilarri et al. 2022). Despite their adaptability to a broad range of environmental conditions, these animals still exhibit sensitivity to the abiotic changes (e.g. in salinity and temperature) (Passos et al. 2016; Souza et al. 2018). Estuarine assemblages, in fact, often exhibit significant spatial and temporal variability (Sheaves 2009; Nagelkerken et al. 2015), emphasizing the importance of long-term datasets to facilitate a comprehensive assessment of fish assemblage dynamics and the influence of environmental drivers on them. Long-term monitoring data may also contribute to advances in invasion biology. In fact, there are few studies on the temporal aspects of invasive species impacts, and most of these studies are of short duration (less than 1 year), making it difficult to develop effective invasive management strategies and implement effective conservation measures (Matsuzaki et al. 2011). In addition, the use of traits can be useful in understanding invasion patterns and predicting which native species are most likely to be vulnerable to invasive species (Matsuzaki et al. 2011).

In this way, a long-term fish assemblage monitoring was conducted in the Minho Estuary (northwestern Iberian Peninsula) from 2010 to 2019. A recent study found that the Minho Estuary fish assemblage has been impacted by climatic changes and extreme temperature (heatwaves and cold-spells) and precipitation (dry and wet) events, which resulted in a less taxonomically diverse fish community dominated by a few invasive species (Ilarri et al. 2022). However, the temporal changes in the species trait composition of the Minho Estuary fish assemblage are still not known. To address this knowledge gap, a decade's worth of data on fish trait composition and climate (temperature and precipitation) from Minho Estuary were compiled from weekly *in-situ* fish sampling and satellite data. We hypothesize that climate change and extreme weather events alter the trait and functional diversity of estuarine fish communities, with most indices of functional diversity (e.g. functional divergence, dispersion, richness, evenness and RAO's quadratic entropy) being expected to decrease, while the functional redundancy is expected to increase. This is due to the increased prevalence of invasive species, which might introduce different traits compared to those originally found in the fish community, but also contribute to the loss or decline of some traits in the fish community.

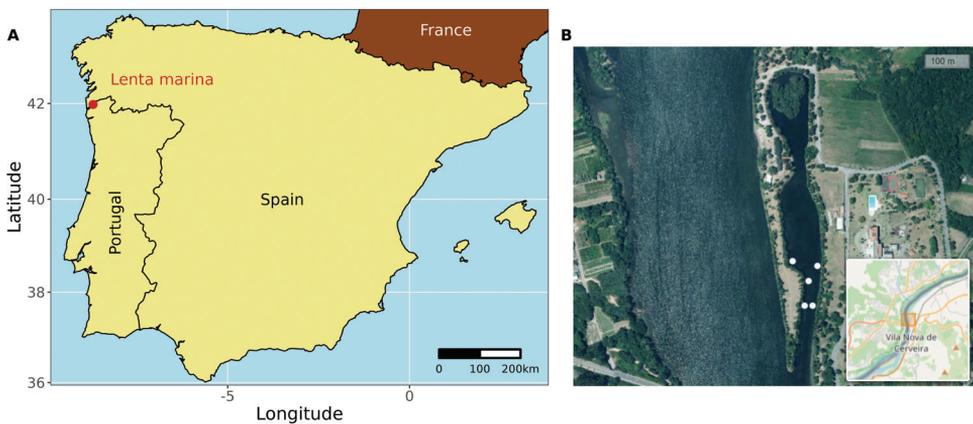
## Methods

### Study area

Sampling took place in Lenta Marina, a small, semi enclosed bay (660 m × 80 m), located 14.5 kilometers upstream in the Minho Estuary (41°57'18.7"N, 8°44'42.9"W) (Fig. 1). Among the estuaries of Portugal, the Minho Estuary has relatively low levels of pollution (Reis et al. 2009), being used as a reference site in toxicology studies (Moreira et al. 2006; Guimarães et al. 2012). The Minho Estuary has also a significant history of biological invasions (Sousa et al. 2008, 2013; Ilarri et al. 2014), nevertheless, the number of invasive species in this area is comparatively lower than in other areas of the Iberian Peninsula (Muñoz-Mas et al. 2021). The Minho Estuary is described as mesotidal, with an average depth of 2.6 meters and a maximum depth of 26 meters (Alves 1997). It is partially mixed, except during flood periods when it tends to exhibit salt wedge conditions (Sousa et al. 2005). During summer or drought events, marine water enters the Lenta Marina as rainfall and water flow decrease (Ferreira et al. 2003). However, despite the occurrence of marine water intrusion, the influence of salinity in the Lenta Marina is relatively small. Salinity values typically range between 0 and 2.0 psu, with higher values observed in the late summer months or during dry periods (Sousa et al. 2013).

### Fish data

Fish samples were collected from January 2010 to November 2019, even though the samples were generally collected on a weekly basis, the actual intervals between samples varied slightly (Souza et al. 2023). The fyke nets were placed in fixed locations near the entrance of the peninsula where the Lenta Marina is harbored (Fig. 1). Double entry



**Figure 1.** Representation of the study area (Lenta Marina) and sampling locations (Fyke nets) in the Minho estuary **A** map of the Iberian Peninsula showing the sampling site in the Minho estuary **B** an enlarged view of the study area highlighting the precise locations of the fyke nets (white circles) within the Lenta Marina.

fyke nets with a mesh size of 10 mm, measuring 7 meters in length, 0.7 meters in mouth diameter, and equipped with a 3.5 meter central wing, were used for the collection. These nets were always deployed in the morning and remained submerged for an average of  $5.7 \pm 3.5$  days (mean  $\pm$  SD). Once the fyke nets were retrieved, all captured fish were identified to the lowest taxonomic level and counted. In total, 3029 samples were collected throughout the study period. The average catch per unit effort (CPUE) per sampling date was determined by dividing the number of individuals caught by the number of days each fyke net remained in the water, taking into account the number of replicates per date. On average,  $4.9 \pm 0.4$  fyke nets were used per sampling date, although this number varied due to technical limitations. A more detailed description of the sampling procedure can be found in a previous study by Ilarri et al. (2022).

### Trait composition

All fish species sampled in the Minho Estuary from January 2010 to November 2019 (more details in Ilarri et al. 2022) were analyzed according to 20 traits (14 biological and 6 ecological) containing 69 modalities (Appendix 1). The specific trait for each species and/or genera was classified following the information presented in the [www.freshwaterecology.info](http://www.freshwaterecology.info) database (see Appendix 2) (Schmidt-Kloiber and Hering 2015), that follows a single category assignment approach for fishes. When the value of any given species and trait was missing, the trait classification was complemented, if possible, with the information present in Cano-Barbacid et al. (2020). When the information of a particular trait modality was missing, NA (not available) was attributed to it, otherwise the values were either zeroes (0) or ones (1). The value used for each trait modality per sample was obtained by the computation of the community-weighted mean (CWM) using the function *dbFD* from the *FD* package in R (Laliberté et al. 2022). The CWM uses the classification of each species into a trait category as previously described and the abundance of species (in our case, CPUE) to compute the values of each modality per sample, which were used for further statistical analysis. In this study, we have compiled the trait composition data from 23 taxa (17 native and 6 non-native) (Table 1).

### Functional diversity indices

Six different functional diversity indices that were often used in previous studies (Villéger et al. 2008, van der Linden et al. 2016) to investigate the effects of environmental disturbances were selected. Functional diversity indices were calculated using the information on fish abundances and their traits classification, namely the functional divergence index (FDiv), the functional dispersion index (FDis), the functional richness index (FRic), the functional evenness index (FEve) (Villéger et al. 2008), Rao's quadratic entropy index (FRAO) (Lepš et al. 2006), and the functional redundancy index (FRed) (de Bello et al. 2007). The first five indices (FDiv, FDis, FRic, FEve and FRAO) were computed using the *dbFD* function from the *FD* package in R (Laliberté et al. 2022), while FRed was interpreted as a normed version of the mean functional similarity (Ricotta et al. 2016). The taxonomic index of diversity (Shannon's diversity

**Table 1.** Origin, family, species, vernacular name, total number of individuals captured (N), first record in the study area (only for the invasive species), and native range (only for the invasive species) of the fishes sampled from January 2010 to November 2019 in the Minho Estuary (Portugal). Fish species are ordered by origin and phylogenetic order (family).

Origin	Family	Species	Vernacular name	N	1 <sup>st</sup> record	Native range
Native	Petromyzontidae	<i>Petromyzon marinus</i>	Sea lamprey	12	–	
Native	Anguillidae	<i>Anguilla anguilla</i>	European eel	2971	–	
Native	Clupeidae	<i>Alosa</i> spp.	Allis and twaite shads	1	–	
Native	Cobitidae	<i>Cobitis paludica</i>	Iberian loach	9031	–	
Native	Leuciscidae	<i>Achondrostoma arcasii</i>	Panjorca	413	–	
Native	Leuciscidae	<i>Pseudochondrostoma duriense</i>	Douro nase	684	–	
Native	Leuciscidae	<i>Squalius carolitertii</i>	Iberian chub	20	–	
Native	Salmonidae	<i>Salmo trutta</i> subsp. <i>fario</i>	Brown trout	162	–	
Native	Salmonidae	<i>Salmo trutta</i> subsp. <i>trutta</i>	Sea trout	259	–	
Native	Atherinidae	<i>Atherina boyeri</i>	Sand smelt	1079	–	
Native	Mugilidae	<i>Chelon auratus</i>	Golden grey mullet	51	–	
Native	Mugilidae	<i>Chelon labrosus</i>	Thicklip grey mullet	28	–	
Native	Mugilidae	<i>Chelon ramada</i>	Thinlip mullet	1581	–	
Native	Mugilidae	<i>Mugil cephalus</i>	Flathead grey mullet	167	–	
Native	Gasterosteidae	<i>Gasterosteus aculeatus</i>	Three-spined stickleback	1221	–	
Native	Moronidae	<i>Dicentrarchus labrax</i>	European seabass	100	–	
Native	Pleuronectidae	<i>Platichthys flesus</i>	European flounder	1207	–	
Invasive	Centrarchidae	<i>Lepomis gibbosus</i>	Pumpkinseed	47302	2000s (Sousa et al. 2008)	ENA
Invasive	Centrarchidae	<i>Micropterus salmoides</i>	Largemouth bass	570	1950s (Antunes 1990)	ENA
Invasive	Tincidae	<i>Tinca tinca</i>	Tench	577	1990s (Antunes and Rodrigues 2004)	EUR
Invasive	Gobionidae	<i>Gobio lozanoi</i>	Iberian gudgeon	255	1990s (Hervella and Caballero 1999)	IBE
Invasive	Cyprinidae	<i>Carassius auratus</i>	Goldfish	20	1950s (Antunes 1990)	ASIA
Invasive	Cyprinidae	<i>Cyprinus carpio</i>	Common carp	3146	1990s (Antunes and Rodrigues 2004)	ASIA, EU

ENA = Eastern North America; EUR = Europe; IBE = Iberian Peninsula; ASIA = Eastern Asia

index) was calculated using the function diversity from the vegan package in R (Ok-  
sanen et al. 2022).

FDiv refers to how trait categories are distributed among individuals (Mason et al. 2005; Villéger et al. 2008). FDiv is low when the most abundant species have trait categories that are near the center of the trait space and high when the most abundant species have extreme trait categories (Mason et al. 2005). FDis measures the mean distance of the individual species from the center of the trait space occupied by the species, it computes the distance of the species from the mean dissimilarity (Villéger et al. 2008, van der Linden et al. 2016). FRic measures the amount of trait space filled by the species in the community. Typically, lower FRic values are associated with communities with similar traits (van der Linden et al. 2016; Maure et al. 2018). FEve measures the evenness of the distribution of the traits' abundance. It is the highest when there is an even distribution of species and abundance of traits (van der Linden et al. 2016). FRAO is an index that measures the trait dissimilarities in the community (Botta-Dukát 2005) and it is conceptually similar to FDis (Laliberté and Legendre 2010). FRed defines the extent

to which a community is saturated with species that have similar traits, with higher values indicating that the community is functionally redundant, while low values indicate that the functional redundancy in the community is low (de Bello et al. 2007).

## Climate data

Climate data used in this study included daily mean air temperature (measured 2 meters above ground level) in °C and precipitation in mm.m<sup>-2</sup>. These data were scaled down to 1×1° grids and covered the entire duration of the sampling campaign, which ranged from January 2010 to November 2019. Data for the sampled site were obtained from NASA via their application programming interface (API) available through the NASA Langley Research Center (LaRC) POWER Project website. The *jsonlite* package in R developed by Ooms et al. (2022) was used to process the data.

Two different categories were used for the identification of extreme temperature events: cold spells and heat waves. The daily averages of air temperature were used to detect and determine the duration and strengthen of these extreme weather events. For this purpose, the *detect\_event* function from the *heatwaveR* package in R, introduced by Schlegel and Smit (2021), was used. To assign a specific category to each climate extreme event, the *category* function from the same package was used, following the methodology described by Hobday et al. (2018).

To analyze precipitation patterns, the standard precipitation index (SPI) was calculated. The SPI quantifies the number of standard deviations by which the observed cumulative precipitation deviates from the climatological mean, as described by McKee et al. (1993). The daily precipitation data were processed using the *spi* function from the *precintcon* package (Povoa and Nery 2016). Based on the SPI values, each date was assigned to one of three precipitation state groups: normal (SPI greater than -1 and less than 1), dry (SPI less than -1) or wet (SPI greater than 1).

## Data analysis

Generalized additive models (GAM) with Gaussian distributions were used to assess the effects of temperature, precipitation and time on the fish trait means and diversity indices. Prior to analysis, the temperature and precipitation data were scaled (i.e. standardized with a mean of zero and a standard deviation of one) using the *scale* function from the *base* package in R (R Core Team 2023). CPUE values, diversity indices and precipitation data were appropriately transformed when necessary. Square root or log( $X + 1$ ) transformations were applied using the *sqrt* and *log1p* functions from the *base* package in R (R Core Team 2023).

For the temperature and precipitation data, cubic regression splines were used to smooth the variables for each season (winter, spring, summer and autumn). This smoothing process was carried out using the function *s* from the package *mgcv* (Wood 2022). The decision to apply smoothing by season was made in view of the different temperature patterns and precipitation profiles observed in each season, which are better captured when the penalty is applied on a seasonal basis.

As the dataset was a time series, the models from GAM included an autocorrelation structure with a lag effect. The initial value for the autocorrelation parameter ( $\rho$ ) was determined by running a GAM model without the autocorrelation structure. The *start\_value\_rho* function from the *itsadug* package (van Rij et al. 2022) was used to calculate the initial value of  $\rho$ . Autocorrelation and partial autocorrelation were evaluated using the *acf* and *pacf* functions from the *stats* package in R package (R Core Team 2023). The GAM models were run with the *bam* function from the *mgcv* package (Wood 2022). All data analyses were performed using the R software, version 4.3.1 (R Core Team 2023).

## Data availability statement

Data used in this study are available for validation and further investigation. The fish occurrences dataset is archived on Zenodo (doi: 10.5281/zenodo.8279744), and detailed trait classification information can be found in Appendix 2. Climate data were sourced from the NASA using an API and a copy of the raw data and its description can be found in Suppl. material 1.

## Results

### Fish assemblage trait composition all over the years

Of the 67 fish traits modalities observed, 65.7% have varied significantly over time (Table 2). Temperature had correlated more strongly with trait modalities than precipitation, 59.7% of the traits modalities responded significantly to temperature, while 23.9% responded significantly to precipitation (Table 2). In addition, temperature correlated to the traits' modalities mostly during winter (59.7%) and autumn (46.3%). Precipitation influenced the trait modalities in a similar pattern, with winter (23.9%) and autumn (16.4%) having greater correlations than summer (7.5%) and spring (0%) (Table 2). Of the 67 GAM models for each trait modality, 19 had a percentage of explanation higher than 50% (Table 2, Fig. 2).

### Traits' composition associated with the invasive and native species

There were some traits' modalities that were more frequently associated with the native species, such as Diadromous (Migration), Litophilic and Other (Reproduction habitat), Freshwater-brackish-marine (Salinity),  $\geq 39$  (Body length), Sh3 (Shape factor) and Winter time (Spawn time) (Fig. 3). On the other hand, it was also observed that other traits modalities were more frequently associated with invasive species such as Potamodromous (Migration), Benthopelagic (Habitat), Phytophilic and Phyto-litophilic (Reproduction habitat), Freshwater and Freshwater-brackish (Salinity),  $> 15$  (Life span), Sh1 (Shape factor), Sw3 (Swimming factor), 3–4 (Female maturity), Summer time (Spawn time),  $\leq 7$  (Incubation period), 55k–60k

**Table 2.** Results of GAM models made to evaluate the effects of temperature, precipitation, and time on the fish assemblage trait composition of the Minho Estuary (Portugal). Statistical significance at: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Win = winter, Spr = spring, Sum = summer, and Aut = autumn. The description of all modalities and their units can be found in Appendix 1.

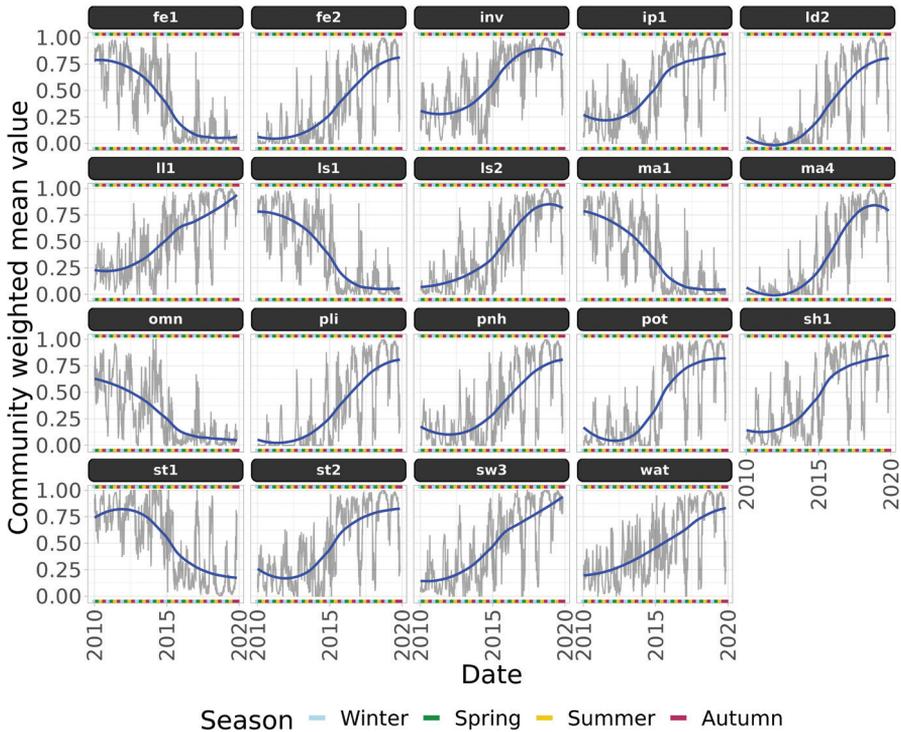
Trait	Modality	Temperature				Precipitation				Time	% exp.	r <sup>2</sup> adj.
		Win	Spr	Sum	Aut	Win	Spr	Sum	Aut	F		
Migration	Nom (No migration)	0.76	0.05	1.74	0.73	0.60	0.05	3.41**	5.80***	-4.39***	15	0.124
	Oce (Oceanodromous)	2.25	3.69*	1.79	0.97	0.00	1.66	1.41	0.01	-1.55	4.6	0.028
	Pot (Potamodromous)	3.57**	0.42	1.63	2.59	1.79	0.00	0.03	4.06*	10.64***	58.3	0.572
	Dia (Diadromous)	4.09**	0.12	2.43	8.93**	0.75	0.52	0.06	1.41	-4.07***	28.7	0.27
Habitat	Ben (Benthopelagic)	6.97***	0.26	1.07	10.86***	0.38	0.62	0.13	0.43	1.64	22.6	0.211
	Dem (Demersal)	3.54*	1.34	0.31	6.67**	1.23	0.18	0.08	0.08	-2.7**	12.9	0.112
	Pel (Pelagic)	12.15***	1.54	1.06	4.20*	6.10***	0.42	0.01	0.23	2.48*	24.5	0.223
Rheophily	Lim (Limnophilic)	9.53**	0.16	1.17	2.74*	1.04	0.84	0.28	0.55	1.81	11.8	0.104
	Eur (Eurytopic)	4.42**	0.17	1.37	6.18*	1.64	0.82	0.23	0.64	0.06	12.1	0.097
	Rhe (Rheophilic)	0.50	0.28	1.52	0.63	0.05	0.00	0.18	0.26	-6.95***	15.9	0.144
Feeding habitat	Benthivorous	3.21*	3.68	0.40	1.34	1.73	0.71	0.83	1.37	-10.93***	49.3	0.48
	Water column	3.01*	4.39*	0.26	1.24	1.39	0.61	0.80	1.26	10.81***	50.8	0.496
Reproduction habitat	Phy (Phytophilic)	3.23*	0.44	0.95	4.63*	13.99***	0.03	1.06	1.47	-1.88	11.1	0.091
	Lit (Litophilic)	2.64	0.07	1.53	2.59	0.03	0.24	0.09	0.30	-5.09***	14.5	0.13
	Phy (Phyto-litophilic)	2.23	2.62	0.61	5.18*	1.10	0.00	0.91	0.69	12.31***	61.8	0.61
	Psa (Psammophilic)	0.27	0.07	1.33	1.12	0.02	1.18	0.05	0.29	-4.64***	10.8	0.091
Salinity	Oth (Other)	1.72	1.53	2.68	5.03*	2.42	0.13	0.21	0.12	-4.81***	12.9	0.114
	Fre (Freshwater)	3.64*	0.00	3.11	7.95**	0.02	1.35	0.46	0.23	3.81***	23.8	0.222
	Frb (Freshwater-brackish)	0.32	0.59	0.06	0.63	3.44	0.08	1.39	3.62*	-1.53	2.4	0.004
	Fbm (Freshwater-brackish-marine)	9.01***	0.01	3.18*	12.22**	2.18*	0.18	0.01	0.29	-3.51***	29.9	0.28
	Fma (Freshwater-marine)	1.31	1.23	0.97	0.03	0.01	1.63	0.05	1.17	-0.40	3.0	0.014
	Brm (Brackish-marine)	0.00	0.07	1.30	7.23***	2.04	0.00	0.00	3.81***	-1.72	14.3	0.117
Feeding diet	Car (Carnivorous)	0.78	1.23	1.33	2.82	1.17	0.47	0.01	2.96*	-3.75***	10.4	0.088
	Inv (Invertivorous)	1.37	1.26	0.18	0.26	0.40	0.68	0.15	0.00	13.44***	58.5	0.577
	Omn (Omnivorous)	0.06	3.28	1.16	0.22	0.04	1.77	0.11	1.11	-11.77***	51.5	0.506
	Oth (Other)	3.42**	0.09	0.01	0.00	1.67	0.04	0.02	0.00	-1.73	4.4	0.023
Life span	<8	2.75*	2.04	1.18	0.00	5.34*	0.51	0.17	0.38	-15.91***	66.7	0.658
	8–15	4.01**	1.40	0.72	3.94*	9.80**	0.08	0.84	0.98	16.63***	70.5	0.699
	>15	0.08	0.05	0.61	5.44*	0.24	0.33	0.32	1.73	-1.06	3.40	0.017
Body length	<=20	3.37	0.13	0.05	1.59	0.84	1.42	0.49	0.99	2.74**	10.6	0.09
	20–39	6.66***	0.96	1.74	0.90	2.20*	1.45	3.84***	4.71*	-2.61**	12.2	0.088
	>=39	1.07	0.23	0.71	7.70**	0.24	0.12	0.30	2.02	-1.31	5.3	0.037
Body shape	Sh1 (<= 4.35)	4.21**	0.63	2.44	7.19**	0.94	0.03	0.77	1.03	11.29***	58.8	0.577
	Sh2 (4.35–4.78)	2.25	3.69*	1.79	0.97	0.00	1.66	1.41	0.01	-1.55	4.6	0.028
	Sh3 (4.78–5.6)	14.07***	2.32	4.61**	4.38*	3.32**	1.40	0.09	0.28	-3.34***	32.9	0.309
	Sh4 (>=5.6)	3.44*	1.06	3.18	9.67**	4.18*	0.29	0.06	0.14	-1.82	11.9	0.103

Trait	Modality	Temperature				Precipitation				Time	% exp.	r <sup>2</sup> adj.
		Win	Spr	Sum	Aut	Win	Spr	Sum	Aut	F		
Swimming factor	Sw1 (Fast swimmer)	0.20	4.06***	5.07**	0.41	0.08	2.02	0.02	0.86	-8.97***	30.0	0.283
	Sw2 (Average swimmer)	13.89***	0.96	3.71	8.69**	0.94	0.15	0.15	0.86	-0.26	26.6	0.251
	Sw3 (Slow swimmer)	9.05***	3.48*	1.69	3.03	3.29	0.02	0.03	0.33	17.84***	69.7	0.69
Female maturity	<=2	4.00**	2.57	3.49*	0.00	5.75*	1.07	2.09*	0.00	-16.68***	66.5	0.655
	2-3	2.69*	0.01	6.84***	0.80	0.27	0.13	7.01***	1.22	-4.17***	19.0	0.162
	3-4	0.16	0.48	0.01	0.94	2.03	0.03	0.64	2.29	-0.63	1.1	-0.006
	4-5	4.12**	1.37	0.00	1.53	9.21**	0.15	0.62	2.34	14.62***	70.7	0.7
	>=5	0.60	1.43	3.06	9.03**	0.40	0.41	0.13	0.33	-1.72	6.7	0.051
Spawn time	Winter time	3.23*	3.61	0.35	2.16	2.85*	1.56	0.01	0.71	-10.24***	51.1	0.499
	Summer time	3.30**	3.53	0.37	1.86	4.01*	1.41	0.03	1.31	10.12***	53.8	0.528
Incubation period	<=7	4.72***	3.41	1.54	5.37*	2.26	0.94	0.18	0.68	10.04***	55.0	0.538
	7-14	3.42**	0.09	0.01	0.00	1.67	0.04	0.02	0.00	-1.73	4.4	0.023
	>14	10.69***	1.68	2.01	1.87	5.35***	0.75	0.03	0.01	0.72	24.1	0.219
Fecundity	<=55k	2.70*	1.98	1.35	0.01	4.57*	0.52	0.27	0.36	-16.74***	67.7	0.668
	55k-60k	1.31	2.27	0.55	3.87	0.56	0.00	0.92	0.78	12.18***	60.3	0.595
	>60k	0.02	0.03	0.01	4.09*	0.06	0.44	0.93	0.91	-1.30	3.5	0.018
Relative fecundity	>=57	0.44	0.03	1.32	0.20	0.13	0.02	1.82	4.80**	-4.71***	14.0	0.12
	57-200	0.14	0.90	0.27	0.38	1.83	0.06	1.43	3.67**	-0.99	1.6	-0.008
	>200	4.70***	0.72	4.85*	4.68*	4.29***	1.36	0.06	0.25	-1.45	18.8	0.165
Egg diameter	<1.35	7.35***	0.80	3.21	4.54*	1.98	0.12	0.30	0.60	6.05***	29.5	0.282
	1.35-2	2.03	0.05	3.47	1.39	11.80***	0.15	0.50	2.79*	-6.98***	25.9	0.244
	>2	6.37***	0.16	3.49	9.91**	0.26	0.06	0.21	0.06	-5.23***	21.4	0.199
Larval length	<=4.2	9.93***	8.81**	0.29	2.23	3.68	0.38	0.45	1.47	15.6***	64.3	0.636
	4.2-6.3	0.30	1.04	0.04	0.22	3.10	0.00	2.03	3.78**	-1.46	2.1	0.001
	>6.3	6.31***	0.16	3.51	9.94**	0.26	0.06	0.21	0.18	-5.26***	21.5	0.199
Parental care	Phn (Protection with nester or eggs hiders)	3.44*	4.61*	0.20	4.44*	1.92	0.99	0.98	0.60	10.65***	57.0	0.561
	Nnh (No protection with nester or eggs riders)	6.99***	0.13	3.88*	10.26**	0.26	0.08	0.18	0.06	-5.42***	22.0	0.205
	Nop (No protection)	9.37**	0.01	0.64	6.35*	0.42	1.15	1.01	0.59	-0.93	11.7	0.099
Larval stage duration	<12	0.17	0.50	2.14	0.02	2.60	0.14	2.29*	5.78***	-2.09*	6.1	0.032
	12-25	2.02	2.66	0.12	2.02	1.50	0.01	0.76	2.05	11.86***	63.0	0.62
	>25	7.27***	0.28	1.25	5.37*	0.78	0.06	0.31	0.02	-5.31***	14.0	0.123

(Fecundity), 57-200 (Relative fecundity), <1.35 and 1.35-2 (Egg diameter), <=4.2 and 4.2-6.3 (Larval length), Nop (No protection) (Parental care) and <12 (Larval stage duration) (Fig. 3).

### Extreme weather events

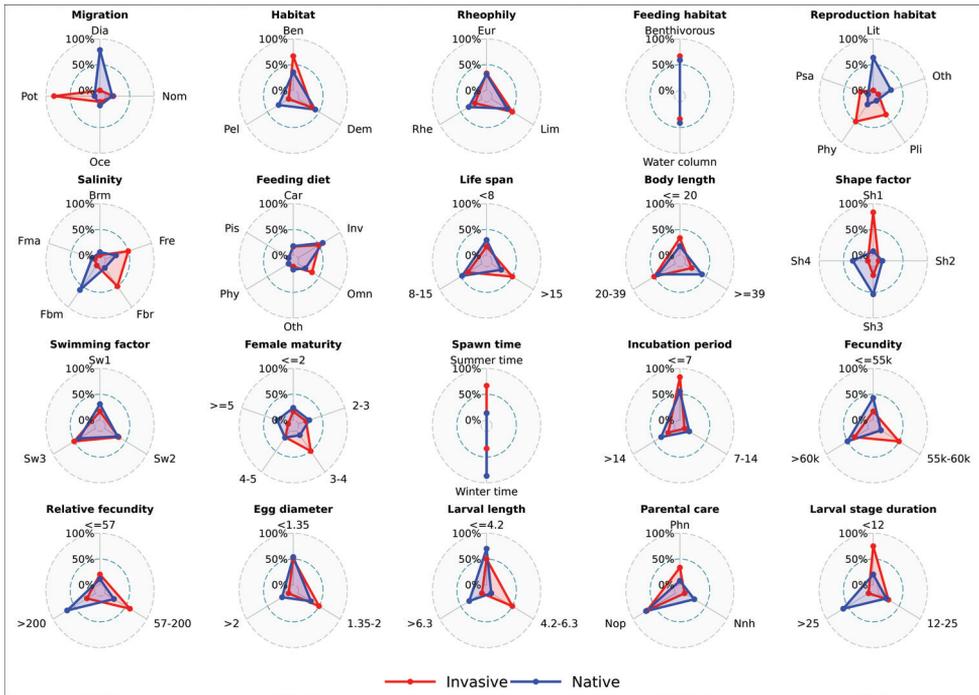
Over the study period, there were 52 extreme temperature events (19 heatwaves: 9 moderate and 10 strong; 33 cold-spells: 32 moderate and 1 strong), and 44 extreme



**Figure 2.** Selection of fish trait modalities in the Minho Estuary (Portugal) that had strong temporal changes (selected by the highest % of variation). Blue lines refer to a simple moving regression (loess) and are only indicative of the temporal changes. Statistical tests can be found in Table 2. fe1 = number of oocytes less or equal to 55000, fe2 = number of oocytes between 55000 and 60000, inv = invertivorous (feed on invertebrates), ip1 = incubation period is less or equal to seven days, ld2 = larval stage duration between 12 and 25 days, ll1 = larval length is smaller or equal to 4.2 cm, ls1 = life span is less than eight years, ls2 = life span is between eight and fifteen years, ma1 = females are mature before two years, ma4 = females are mature between four and five years, omn = omnivorous (feed on animals and plants), pli = reproduction habitat is phyto-litophilic (associated with plants and rocks), pnh = parental care by protection with nesting or egg hiding, pot = potamodromous migration (between different freshwater bodies), sh1 = shape factor ratio is smaller or equal to 4.35 (compact, rounded body shape), st1 = spawn time is during winter, st2 = spawn time is during summer, sw3 = slow swimmer, wat = feeding habitat is in the water column. The description of all modalities and their units can be found in Appendix 1.

precipitation events (21 dry and 23 wet) in the area of the Minho Estuary sampled. For more details see Ilarri et al. (2022).

The moderate heatwave events correlated positively and negatively with some trait modalities, and of these, only 1.5% of the traits had a strong decrease in their mean value during these kind of extreme events (e.g., Salinity: Brackish-marine, decrease of 100%), and about 4.5% had a strong increase (Migration: Oceanodromous, increase of 316%; Shape factor: 4.35–4.78, increase of 316%; Reproduction habitat: Psammophilic, increase of 117%) (Table 3).



**Figure 3.** Comparison between invasive and native fish species at the Minho Estuary (Portugal) on the average score of each trait modality. The description of all modalities and their units can be found in Appendix 1.

Regarding the strong heatwave events, 31.3% of the traits had a strong decrease during these events (e.g., Egg diameter: >2, decrease of 100%; Habitat: Pelagic, decrease of 100%), and 4.5% had a strong increase (e.g., Migration: Oceanodromous, increase of 291%; Shape factor: 4.35–4.78, increase of 291%) (Table 3).

Over 6% of the traits had a strong decrease in their values during moderate cold-spells’ events (e.g., Incubation period: 7–14, decrease of 100%; Salinity: Brackish-marine, decrease of 100%), while about 4.5% of the trait modalities had a strong increase during these extreme events (Migration: Oceanodromous, increase of 450%; Shape factor: 4.35–4.78, increase of 450%) (Table 3).

During the dry events recorded in the Minho Estuary between 2010 and 2019, there was no trait negatively affected by more than >=70.01% of their mean value (Table 3). Only 4.5% of the trait modalities experienced a strong increase in their mean value (e.g., Incubation period: 7–14, increase of 139%; Feeding diet: Other, increase of 139%; Salinity: Brackish-marine, increase of 96%) during dry events (Table 3).

On the other hand, the wet events contributed to strong decreases, 10.4% of the trait modalities were negatively influenced (e.g. Incubation period: 7–14, decrease of 100%; Feeding diet: Other, decrease of 100%) (Table 3). On the other hand, there was no trait positively affected by more than >=70.01% of their mean value during the wet events (Table 3).

**Table 3.** Categorical representation of the influence of the extreme climatic events (temperature heat-waves: moderate and strong, and temperature cold-spells: moderate; precipitation: dry and wet) on the traits modalities of the fish assemblage of the Minho Estuary (Portugal). The traits classification was made considering the traits mean values per event. Classification as: 0 refers to change of  $\pm 10\%$  in the traits mean values during the event compared to the mean values during the normal conditions; + refers to an increase in the traits mean values from 10.01 to 40%; ++ refers to an increase in the traits mean values from 40.01 to 70% , +++ refers to an increase in the traits mean values abundance  $\geq 70.01\%$ ; - refers to a decrease in the traits mean values from 10.01 to 40%; -- refers to a decrease in the traits mean values from 40.01 to 70% , --- refers to a decrease in the traits mean values  $\geq 70.01\%$ . The description of all modalities can be found in Appendix 1.

Trait	Modality	Temperature			Precipitation	
		Heatwave		Cold-spells	Dry	Wet
		Moderate	Strong	Moderate		
Migration	Nom (No migration)	--	---	+	-	++
	Oce (Oceanodromous)	+++	+++	+++	--	---
	Pot (Potamodromous)	+	++	0	0	+
	Dia (Diadromous)	-	---	-	0	-
Habitat	Ben (Benthopelagic)	0	+	0	0	0
	Dem (Demersal)	-	--	0	0	0
	Pel (Pelagic)	++	---	--	+	---
Rheophily	Lim (Limnophilic)	0	+	0	0	0
	Eur (Eurytopic)	+	--	0	-	0
	Rhe (Rheophilic)	0	-	-	0	0
Feeding habitat	Benthivorous	0	-	0	0	-
	Water column	0	+	0	0	0
Reproduction habitat	Phy (Phytophilic)	+	--	-	-	0
	Lit (Litophilic)	-	-	+	0	0
	Phy (Phyto-litophilic)	+	++	0	0	+
	Psa (Psammophilic)	+++	---	---	++	---
	Oth (Other)	-	--	0	-	+
Salinity	Fre (Freshwater)	0	+	0	0	0
	Frb (Freshwater-brackish)	++	-	+	-	+
	Fbm (Freshwater-brackish-marine)	-	---	-	0	-
	Fma (Freshwater-marine)	-	---	0	+	---
	Brm (Brackish-marine)	---	---	---	+++	+
Feeding diet	Car (Carnivorous)	-	---	+	0	++
	Inv (Invertivorous)	+	+	0	0	0
	Omn (Omnivorous)	-	0	0	+	-
	Oth (Other)	--	---	---	+++	---
Life span	<8	-	-	-	0	-
	8–15	+	++	0	0	0
	>15	+	--	+	0	+
Body length	$\leq 20$	0	+	0	0	0
	20–39	0	--	0	0	-
	$\geq 39$	+	--	+	0	++
Body shape	Sh1 ( $\leq 4.35$ )	+	++	+	0	+
	Sh2 (4.35–4.78)	+++	+++	+++	--	---
	Sh3 (4.78–5.6)	0	---	--	0	--
	Sh4 ( $\geq 5.6$ )	-	---	0	0	+
Swimming factor	Sw1 (Fast swimmer)	-	---	--	-	--
	Sw2 (Average swimmer)	+	---	-	-	-
	Sw3 (Slow swimmer)	+	++	+	0	+

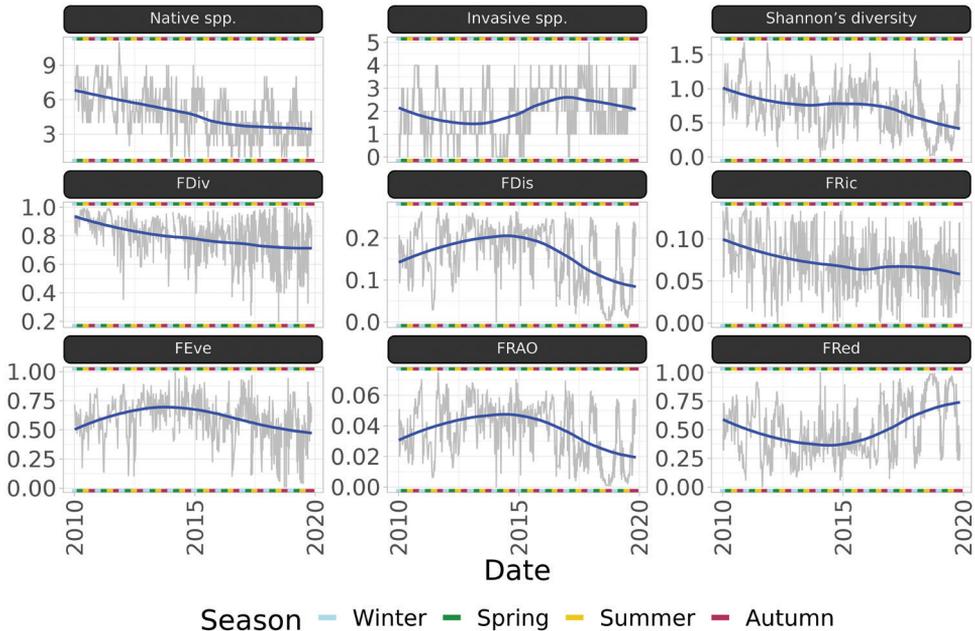
Trait	Modality	Temperature			Precipitation	
		Heatwave		Cold-spells	Dry	Wet
		Moderate	Strong	Moderate		
Female maturity	<=2	-	--	-	+	-
	2-3	-	-	+	-	0
	3-4	++	-	+	-	+
	4-5	+	++	0	0	0
	>=5	-	--	+	0	++
Spawn time	Winter time	-	-	0	0	-
	Summer time	+	+	0	0	+
Incubation period	<=7	+	+	0	-	+
	7-14	--	---	---	+++	---
	>14	++	---	--	+	--
Fecundity	<=55k	-	-	-	0	-
	55k-60k	+	++	0	0	+
	>60k	+	-	+	-	+
Relative fecundity	>=57	--	---	++	0	+
	57-200	++	-	0	-	+
	>200	0	---	-	0	-
Egg diameter	<1.35	0	+	0	0	0
	1.35-2	-	---	0	-	0
	>2	-	---	-	+	+
Larval length	<=4.2	0	+	0	0	+
	4.2-6.3	++	-	0	-	+
	>6.3	-	---	-	+	+
Parental care	Phn (Protection with nester or eggs hidiers)	0	++	0	0	+
	Nnh (No protection with nester or eggs hidiers)	-	---	-	+	+
	Nop (No protection)	+	--	0	0	0
Larval stage duration	<12	+	-	+	-	+
	12-25	+	+++	0	0	+
	>25	+	-	+++	+	-

### Taxonomic composition and functional metrics of the fish assemblage

Over the years, there was a significant reduction in the number of native species, a significant increase in the number of invasive species, and a significant decrease in the taxonomic diversity of the fish community of the Minho Estuary. Significant changes in the functional diversity indices were also recorded for all indices calculated. FDiv, FDis, FEve, FRic, and FRAO decreased significantly through time, while FRed have increased over the years (Fig. 4, Table 4).

### Discussion

We have been monitoring the fish populations in the Minho Estuary for over a decade to better understand the effects of changing environmental conditions on biodiversity. During this period, we have observed signs of decline in both taxonomic and functional diversities, which seems to correspond to a decreasing number of native species and an increasing prevalence of invasive species. This phenomenon seems to be further influenced by changes in environmental factors such as temperature and precipitation,



**Figure 4.** Dynamics of the number of native and invasive species, taxonomic diversity (Shannon's diversity) and six functional diversity indices (FDiv, FDis, FRic, FEve, FRAO and FRed) computed with weekly data on the fish captured by fyke nets in Minho Estuary (Portugal) from 2010 and 2019. Blue lines refer to a simple moving regression (loess) and are only indicative of the temporal changes. Statistical tests can be found in Table 4. FDiv = functional divergence, FDis = functional dispersion, FRic = functional richness, FEve = functional evenness, FRAO = Rao's quadratic entropy and FRed = functional redundancy.

which appear to impact several key trait characteristics of these fishes. Overall, there has been a significant shift in fish assemblage occurring in this estuary over the past decade, which now has an almost equal contribution of native and invasive species in terms of species richness, whereas the latter dominate in terms of abundance (Ilarri et al. 2022).

Over the past few years, the Minho Estuary has witnessed a significant increase in populations of invasive species (Souza et al. 2013; Ilarri et al. 2022). The pumpkinseed, in particular, common carp, and tench are three species that have flourished in the estuary since 2015–2016 (Ilarri et al. 2022), and their increase is likely due to changes in the prevalent environmental conditions. Cano-Barbacid et al. (2022) and Bae et al. (2018) propose temperature as a central factor in explaining the spread of invasive species in the Iberian Peninsula. They emphasize this proposition by pointing out the strong correlation between temperature and thermophilic characteristics of most invasive species, as those from the aforementioned species. Additionally, the pumpkinseed, common carp, and tench prefer slow currents (Benito et al. 2015; Avlijaš et al. 2018; Lages et al. 2021) and highly vegetated zones (Penne and Pierce 2008; Top et al. 2016; Avlijaš et al. 2018), which are likely to become more prevalent with changes in temperature (increase) and rainfall regime (decrease) over time. These three species are also potamodromous, meaning that they perform migrations in the river. In addition,

**Table 4.** Summary of the GAM models with the functional diversity indices calculated with the fish abundances from the Minho Estuary (Portugal) and temperature, precipitation and time. Statistical significance at: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . FDiv = functional divergence index, FDis = functional dispersion index, FRic = functional richness index, FEve = functional evenness index, FRAO = Rao's quadratic entropy index, FRed = functional redundancy index, Win = winter, Spr = spring, Sum = summer, and Aut = autumn.

Index	Temperature				Precipitation			Time	% exp	$r^2$ adj	
	Win	Spr	Sum	Aut	Win	Spr	Aut	F			
# of native species	10.32***	1.64	2.46	20.80***	3.01*	2.28	6.14*	1.87	-11.23***	38.8	0.374
# of invasive species	1.02	1.65	4.24*	0.70	2.76*	0.89	0.04	2.16	4.44***	13.1	0.113
Shannon diversity	2.55	0.70	0.13	0.98	2.38*	0.01	1.60	0.81	-4.25***	18.0	0.170
FDiv	1.28	1.41	3.19	0.81	1.08	0.06	0.03	0.32	-8.10***	16.3	0.147
FDis	2.89*	0.63	0.54	0.33	5.84***	0.67	0.70	0.00	-5.08***	23.9	0.221
FRic	1.29	1.09	0.52	11.85**	0.02	0.03	0.52	0.93	-5.26***	11.8	0.102
FEve	2.55	1.67	0.15	6.05*	0.51	1.52	3.04	0.03	-3.85***	10.8	0.093
FRAO	3.56*	0.87	0.98	0.83	6.52***	0.94	0.52	0.00	-4.99***	22.5	0.206
FRed	3.96**	0.14	0.48	0.68	5.65***	0.52	0.47	0.05	4.37***	20.2	0.183

they are either phytophilic or phyto-litophilic species, reproducing in areas rich in submerged vegetation and rocks. These species are also either eurytopic (common carp) or limnophilic (pumpkinseed and tench), which means that the common carp tolerates a wide range of environmental conditions, while pumpkinseed and tench are associated with slow moving waters. Interestingly, these three species also have the shape 1 classification in terms of body shape (more rounded and compacted body) and are either average (common carp) or slow swimmers (pumpkinseed and tench) (Schmidt-Kloiber and Hering 2015). The traits' characteristics of these three invasive species seem to have been benefited in the Minho Estuary, as the decrease in precipitation and drought events have contributed to reduced river inflow and water currents in the system. Haubrock et al. (2021) also observed a significant increase over time in short-bodied species with high body depth (shape factor 1) on the Arno River (Italy). According to Vila-Gispert et al. (2005), this trait modality can be advantageous when competing with native species in slow-flow waters. On the other hand, the lower river inflow and water currents are not good for many native species with elongated body shapes that are more associated with fast-flowing waters (rheophilic) and oceanodromous or diadromous migration modalities, such as eel, shad, three-spined stickleback, European seabass and sea trout. These species have declined sharply over time (Ilarri et al. 2022), and the traits associated with them are also disappearing from the system.

Interestingly, there was a decline in FRic, and an increase in FRed. This suggests that the fish assemblage is losing some traits and that the invasive species are not able to replace the losses of these traits. This is somewhat expected, as invasive species usually differ from native species in their life-history and ecological traits (Vila-Gispert et al. 2005). However, for the vast majority of traits analyzed in the present study, invasive species had very similar modalities to native species, with an important exception of a few traits. One of the most striking differences is probably observed in migration, where the native species have a good amount of diadromous species and almost all

invasive species are potamodromous. This result highlights that climate change is indeed seriously threatening diadromous species (Limburg and Waldman 2009; Mota et al. 2016; Braga et al. 2022; de Eyto et al. 2022), putting additional pressure on this group, which is already heavily impacted (Barbarossa et al. 2020; Duarte et al. 2021; Podda et al. 2022). For diadromous species in the Minho River, in addition to climate change, additional factors such as an increasing number of dams have exacerbated their decline. The work of Azeiteiro et al. (2021) highlights a notable reduction in Allis shad populations due to the increased dam count in the river. The decline of these species is further evident in the significant shift in trait modalities related to salinity preference. In particular, our results show a decline in species that prefer marine and brackish waters, accompanied by a replacement by species that prefer freshwater environments. It is widely reported that climate change may favor marine and brackish water species in this estuary, at least in the near-term (Souza et al. 2018, 2022a). However, this may not be true for all species, as observed in this study. The sampled area is in the upper part of the estuary, where the saline intrusion is historically not so strong, ranging from 0–2.0 psu during late summer month or droughts period (Souza et al. 2013). Despite the decrease in the river inflow and the precipitation regime, it looks like that the saline intrusion is not affecting much the upper estuary, with the exception of European seabass, which in some years can reach the upper parts of the river in summer due to higher saline intrusion (Ilarri et al. 2022). On the contrary, the change in hydrologic conditions seems to favor freshwater species that prefer slow currents or standing waters (limnophilic or eurytopic), which are also invasive (common carp, goldfish, largemouth bass, pumpkinseed, and tench). This might be explained by the decreased hydrodynamics in the area, which started to attract species with affinity to slow moving freshwater.

Another important divergence in trait modality composition between native and invasive species is in the reproduction habitat. Redundancy in this trait is low, with native species preferring to spawn in rocky areas (litophilic species), while invasive species are more associated to densely vegetated areas (phytophilic species) with some rocky bottoms (phyto-litophilic) or in sandy areas (psammophilic species). The decreased rainfall and river inflow probably contributed to the growth of submerged vegetation and the accumulation of finer substrate (sand) in the area. These conditions are also likely behind the invasion success of the aquatic plant *Egeria densa* in the Minho Estuary, which became very abundant after 2015 (authors' personal observation). A change in the phenology of fish species was also observed. Previously, most species had a spawning season associated with the winter season, but with the increase of invasive species in the area, there has been a change in this trait with an increase in the occurrence of species that have a summer spawning season. Fujiwara et al. (2022) also observed an increasing pattern in non-winter spawners and a decreasing trend in winter spawners when analyzing the temporal patterns of estuarine fish communities from the northwestern Gulf of Mexico. Along with the increase in summer spawning species, a reduction in the incubation period (increase in modality less or equal to seven days) was also a feature introduced by the invasive species now present in the area.

Another trait that showed important divergence between native and invasive species is the life span, with native species having a shorter life span than invasive species. This result is interesting as it is largely recognized that successful invasive species have short life spans (e.g. Jaspers et al. 2018), but this may be different for freshwater fish species in the Iberian Peninsula (e. g. Vila-Gispert et al. 2005). In this region, many of the invasive aquatic organisms arrived several centuries ago and were influenced in the past by the wishes of the rulers of society (monarchs), who deliberately introduced species from Central Europe (Clavero 2022). This important remark is necessary because the characteristics of the traits of the invasive species currently found in the studied system may not be initially selected by the environment, but by men attempting to create an ecosystem similar to that observed in Central Europe. Of the invasive fish species recorded in our study, two originate from North America (largemouth bass and pumpkinseed), while the common carp, goldfish, and tench originate from Eurasia, and the Iberian gudgeon is native to other areas of the Iberian Peninsula but not to the Minho Estuary. Another noteworthy aspect regarding invasive species in the Minho Estuary is that a significant proportion of them consists of species targeted by recreational fisheries, which, usually have different traits when compared with their native counterparts. The introduction of these species follows a meticulous selection by humans aimed at propagating certain desirable traits for angling activities, notably larger body size and a wide ecological tolerance (Alcaraz et al. 2005; Grabowska and Przybylski 2015). Unfortunately, the illegal introduction of species targeted by recreational fishermen in the Iberian Peninsula is still a problem (Clavero and Hermoso 2011).

The traits of fish species are influenced by environmental conditions and are therefore good predictors of how fish species will respond to different climate change events (Winemiller and Rose 1992; Dahlke et al. 2020). The effects of extreme weather events on fish species varies from species to species, probably related to the sensitivity of each species to the type and intensity of the event. Overall, extreme weather events had mostly strong negative effects on fish traits modalities than positive ones (i.e. decreases on the values of trait modalities were more frequent than increases). In our study, heatwaves had the greatest impact on traits compared to the other extreme events. Indeed, Barbarossa et al. (2021) suggest that increases in water temperature constitute a larger threat to freshwater fishes than changes in high and low flow conditions. The heatwaves caused a decline in trait modalities associated with higher salinity preference, reproduction in sandy habitats (Psammophilic), longer body (species with higher shape factors), average and fast swimmers, longer incubation period, low fecundity, high egg diameter, and longer larval length. On the other hand, it was observed a total benefit for short-bodied species. Our results are in part corroborated by Fujiwara et al. (2022), that suggested that fish species sensitive to changes in temperature, generally have traits associated with longer generation time, maximum length and length at maturity. In our study, traits associated with these aspects negatively responded to extreme temperature events. Interestingly, these trait modalities were also negatively correlated with the long-term effects of temperature. Therefore, the heatwave events (especially the strong ones) are possibly accelerating the speed of change in the fish community in Minho Estuary.

Regarding extreme precipitation weather events, both dry and wet events can be critical in estuarine ecosystems due to the hydrological dynamics of these systems. Although the extreme dry events correlated with a large number of species (Ilarri et al. 2022), these events seem to have a broad effect on the whole fish community, with fish traits benefiting more than being negatively affected (mainly considering cases where there was a change in abundance  $\geq 70.01\%$ ). This result differs from our expectations, as we expected that these conditions have mainly negative impacts on the fish functional diversity. Normally, extreme dry events are associated with an increase in salinity and changes in other water biochemical properties (Martinho et al. 2007; Kinard et al. 2021). In this case, salinity and water quality act as abiotic filters in the fish assemblage and select fishes with traits better adapted to harsh conditions (Kinard et al. 2021). Overall, drought events were linked to an increase in the abundance of trait modalities associated with the marine environment (brackish-marine), which was expected as the decreasing water flow can lead to stronger saltwater intrusion into the upper parts of the estuary. Drought events also positively correlated with some traits modalities related to reproduction, such as incubation period, egg diameter and larval length. The favored modalities are not in the extremes of the ranges of the traits, suggesting that they might be indicative of moderate and stable environments, which also suggested that drought events probably did not cause severe stress to the fishes in Minho Estuary. On the other hand, the extreme wet events, despite of affecting a lower number of species than the extreme dry events (see Ilarri et al. 2022), they affect negatively several trait modalities. This result was also different than expected, as areas with more precipitation are normally expected to create more stable conditions than areas submitted to dry conditions. In this sense, wet events can be expected to affect the extreme modalities of traits, and to favor the moderate modalities of traits, which was not the case for several traits in this study. The extreme wet events were mostly linked to a decline in traits associated with the marine environment, such as oceanodromous and freshwater-marine modalities, which makes sense given the lower saline influence under this condition. Other traits' modalities that were negatively correlated were pelagic, psammophilic, shape 2 and incubation period of 7–14 (intermediary modality).

Some studies indicate that changes in functional diversity are easier to detect than changes in taxonomic diversity and serve as early warning signals for threatened ecosystems. However, in this study it was possible to see the same signal in both metrics, suggesting that in the Minho Estuary the deterioration of taxonomic and functional diversity occurred simultaneously. Each functional diversity index provided a different perspective on the functional change that is occurring in the system. For example, the decrease in the FDiv index indicates that some of the most abundant species in the system nowadays have highly convergent characteristics, while FDis, FRic and FRAO tell more or less the same story, namely that the fish assemblage is losing trait richness and diversity, and particularly rapidly after 2015, a period when the dominance of a few invasive species increased significantly. The FRed index, which is a potential early warning indicator of increasing disturbances in the system (van der Linden et al. 2016), shows that the fish assemblage is becoming more functionally redundant. This

result may indicate two different things: first, that some traits that were present, but not dominant are being lost; and second, that the remaining traits are more similar to each other, which may provide some resilience to the assemblage in terms of functional stability (van der Linden et al. 2016).

## Conclusion

The findings of this study demonstrate the negative impacts of climate change and extreme weather on fish communities in estuarine ecosystems. The decline in both taxonomic and functional diversity suggests a threat to the overall balance and health of the ecosystem. These changes show no signs of slowing down, highlighting the need for immediate and effective action to mitigate environmental damage caused by climate change. Furthermore, this loss in fish diversity has implications for local cultures and economies that rely on fish as a source of food and income. It is therefore crucial to address climate change before further harm is inflicted on fish communities and the humans they support.

## Author contributions statement

Conceptualization: ATS, MI. Data curation: ATS, MI. Formal analysis: ATS, MI. Investigation: ATS, CA, ED, MI. Methodology: ATS, CA, MI. Project administration: CA. Resources: CA. Software: ATS, MI. Validation: ATS, CA, ED, MI. Visualization: ATS, MI. Writing original draft: ATS, MI. Writing, review and editing: CA, ED.

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## Appendix I

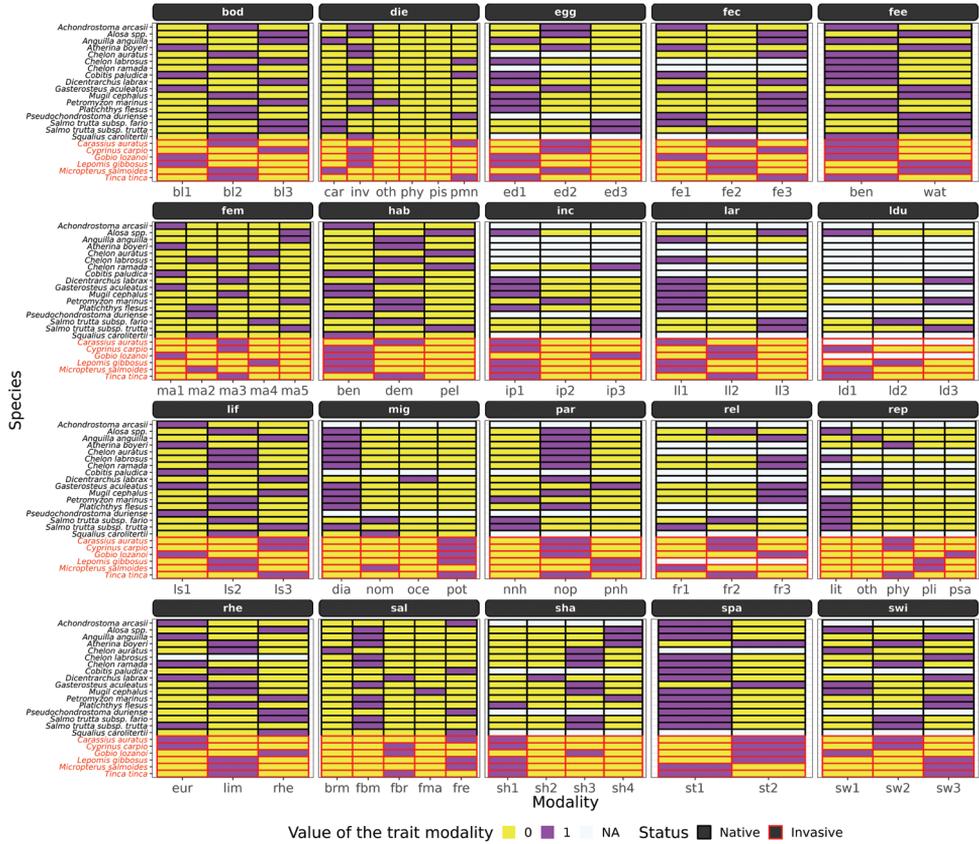
**Table A1.** Fish traits and modalities descriptions based on the information contained in the [www.freshwaterecology.info](http://www.freshwaterecology.info) database.

Trait category	Trait	Trait abbreviation	Modality	Modality description
Biological	Body length	bod	bl1	Smaller or equal to 20 cm
			bl2	Between 20 and 39 cm
			bl3	Larger or equal to 39 cm
Biological	Egg diameter	egg	ed1	Smaller than 1.35 mm
			ed2	Between 1.35 and 2 mm
			ed3	Larger than 2 mm
Biological	Fecundity (# of oocytes)	fec	fe1	Less or equal to 55000
			fe2	Between 55000 and 60000
			fe3	More than 60000
Biological	Feeding diet	die	car	Carnivorous
			inv	Invertivorous
			omn	Omnivorous
			oth	Other
			pis	Piscivorous
			phy	Phytophagous
Biological	Female maturity	fem	ma1	Before 2 years
			ma2	Between 2 and 3 years
			ma3	Between 3 and 4 years
			ma4	Between 4 and 5 years
			ma5	After 5 years
Biological	Incubation period	inc	ip1	Less or equal to 7 days
			ip2	Between 7 and 14 days
			ip3	More than 14 days
Biological	Larval length	lar	ll1	Smaller or equal to 4.2 cm
			ll2	Between 4.2 and 6.3 cm
			ll3	Larger than 6.3 cm
Biological	Duration of larval stage	ldu	ld1	Less than 12 days
			ld2	Between 12 and 25 days
			ld3	More than 25 days

<b>Trait category</b>	<b>Trait</b>	<b>Trait abbreviation</b>	<b>Modality</b>	<b>Modality description</b>
Biological	Life span	lif	ls1	Less than 8 years
			ls2	Between 8 and 15 years
			ls3	More than 15 years
Biological	Parental care	par	nnh	No protection with nester or egg hiders
			nop	No protection
			pnh	Protection with nester or egg hiders
Biological	Relative fecundity <sup>1</sup>	rel	fr1	Less or equal to 57
			fr2	Between 57 and 200
			fr3	More than 200
Biological	Shape factor <sup>2</sup>	sha	sh1	Ratio smaller or equal to 4.35
			sh2	Ratio between 4.35 and 4.78
			sh3	Ratio between 4.78 and 5.6
			sh4	Ratio larger than 5.6
Biological	Spawn time	spa	st1	Winter time
			st2	Summer time
Biological	Swimming factor	swi	sw1	Fast swimmer
			sw2	Average swimmer
			sw3	Slow swimmer
Ecological	Feeding habitat	fee	ben	Benthivorous
			wat	Water column
Ecological	Habitat	hab	ben	Benthopelagic
			dem	Demersal
			pel	Pelagic
Ecological	Migration	mig	dia	Diadromous
			nom	No migration
			oce	Oceanodromous
			pot	Potamodromous
Ecological	Reproduction habitat	rep	lit	Lithophilic
			oth	Other
			phy	Phytophilic
			pli	Phyto-litophilic
			psa	Psammophilic
Ecological	Rheophily	rhe	eur	Eurytopic
			lim	Limnophilic
			rhe	Rheophilic
Ecological	Salinity	sal	brm	Brackish-marine
			fbm	Freshwater-brackish-marine
			fbr	Freshwater-brackish
			fma	Freshwater-marine
			fre	Freshwater

<sup>1</sup>Maximum number of oocites per 100g <sup>2</sup>Total length divided by maximum body depth.

## Appendix 2



**Figure A1.** Values of the modalities of each trait from the fish species captured in the Minho Estuary by fyke nets throughout the course of a decade (2010–2019). Modality classification was based on the information contained in [www.freshwaterecology.info](http://www.freshwaterecology.info) database and complemented by the information present on Cano-Barbacid et al. (2020). For the description of traits and modalities see Appendix 1.

## Supplementary material I

### **Daily air temperature and precipitation data, extracted from the NASA Langley Research Center (LaRC) POWER Project website**

Authors: Allan T. Souza, Ester Dias, Carlos Antunes, Martina Ilarri

Data type: csv

Explanation note: The data ranges from 2010-01-01 to 2019-12-31 (yyyy-mm-dd).

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Link: <https://doi.org/10.3897/neobiota.88.108283.suppl1>