RESEARCH ARTICLE



# Assessing the relative importance of human and spatial pressures on non-native plant establishment in urban forests using citizen science

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#### Abstract

Since 2007, more people in the world live in urban than in rural areas. The development of urban areas has encroached into natural forest ecosystems, consequently increasing the ecological importance of parks and fragmented forest remnants. However, a major concern is that urban activities have rendered urban forests susceptible to non-native species incursions, making them central entry sites where non-native plant species can establish and spread. We have little understanding of what urban factors contribute to this process. Here we use data collected by citizen scientists to determine the differential impacts of spatial and urban factors on non-native plant introductions in urban forests. Using a model city, we mapped 18 urban forests within city limits, and identified all the native and non-native plants present at those sites. We then determined the relative contribution of spatial and socioeconomic variables on the richness and composition of native and non-native plant communities. We found that socioeconomic factors rather than spatial factors (e.g., urban forest area) were important modulators of overall or non-native species richness. Non-native species richness in urban forest fragments was primarily affected by residential layout, recent construction events, and nearby roads. This demonstrates that the proliferation of non-native species is inherent to urban activities and we propose that future studies replicate our approach in different cities to broaden our understanding of the spatial and social factors that modulate invasive species movement starting in urban areas.

#### **Keywords**

biological invasions, citizen science, city planning, non-native plants, urban forests

# Introduction

Contemporary non-native species introductions and dispersal are intimately associated with human activity (Elton 2020) and, as we could expect, these species tend to be adapted to human-modified environments such as urban environments (van Kluenen et al. 2015). While preserving forest remnants within cities can be vastly beneficial (Alvey 2006), urban area expansion also reduces and fragments habitats, which can facilitate invasion by non-native species (Marzluff 2005; Catford et al. 2011).

The type of land use within the urban matrix represents a primary pathway for the introduction of non-native plants. For example, cities contain numerous individually managed gardens, as well as vacant unmanaged land, degraded sites and intensively managed parks where non-native species often abound. This increases the probability of their escape into urban forests (Andrén 1994), which in virtue of their disturbance, fragmentation and isolation from the original main forested area, offer, often times, suitable habitats for non-native species to establish and grow in abundance (Potgieter and Cadotte 2020). Consequently, urban forest planning and management practices should incorporate the unique attributes of urban forests and detach, to some degree, from practices specifically used in natural forests. However, a major challenge is that our understanding of invasion patterns in urban forests is lacking (Cadotte et al. 2017).

Urban forest fragments in a matrix of urbanization can, to a certain extent, be seen as akin to islands, where non-native species establishment may more readily occur due to their relative isolation and fragmentation (Davis and Glick 1978). In this context, we can formulate hypotheses to identity factors driving urban forest plant community composition. These can include, for example, the species-area relationship, which would suggest that the size of an urban forest relates to species richness (MacArthur and Wilson 1963, 1967). In this context, large urban forests can conserve higher native species diversity than smaller ones (Honnay et al. 1999), thereby being relatively more resistant to non-native species introductions than smaller ones. Conversely, edge environments are more prone to non-native species establishment; as a consequence, small urban forests can host more non-native species than their larger counterparts. Indeed, smaller natural forest fragments have previously been shown to have higher levels of invasion (Ohlemüller et al. 2006).

Spatial arrangement (e.g., distance and connectivity) among urban forests can also play a role in the susceptibility of urban forests to non-native species introductions and consequent invasions. For example, it can by hypothesized that the degree of isolation among urban forest fragments affects species richness with consequences for non-native plant richness (MacArthur and Wilson 1963, 1967; see Bastin and Thomas 1999). If this is the case, conservation measures relating to urban forest fragments and biodiversity may be informed if a distance effect among urban forests is noticeable. Furthermore, the relatively small size and proximity of urban forests to areas of high human traffic and activity makes them vulnerable to edge effects (Murcia 1995), and non-native species incursions (Ohlemüller et al. 2006). Thus, native and non-native species composition of urban forests can be influenced by both spatial variables and attributes associated with the surrounding urban matrix (Kupfer et al. 2006). For example, non-native plant species can be abundantly brought into the urban matrix either intentionally (e.g., garden ornamentals, bird seeds) and unintentionally (e.g., via increased vectors and pathways), and thus exert a high propagule pressure on urban forests compared to native forest species that must traverse the urban matrix to reach the same forests (Nascimento et al. 2006). In fact, urban forests have more non-native species when located closer to the center of urban cores (Mack and Lonsdale 2001; Kowarik 2008). Additionally, native species within urban forests may experience greater rates of population declines the further apart they are from the nearest original and/ or unfragmented forest (Gascon et al. 1999). Whether this is the result of direct and indirect interactions with non-native species or results from reproductive isolation is unknown. As such, elucidating what urban forest features contribute to non-native species richness can contribute to answer this question.

One can hypothesize that the type of land use, and the socioeconomic urban layout can be predictors of non-native species richness in urban forests. Both species richness and propagule pressure from non-native species brought into the urban matrix can vary from one neighborhood to another (Aronson et al. 2015). For example, Fan et al. (2019) studied the effects of urban landscape variables on forest community structure in Illinois, and found that while industrial, commercial and transportation land use decreased the diversity and canopy cover of trees, residential land use had a positive effect on those variables. Therefore, land use must be considered when assessing the factors determining species composition and invasion patterns in urban forests. Certainly, the relative importance of spatial factors and urban matrix factors remains unclear.

A limitation of urban studies is associated with their inherent complex spatio-temporal scales (Ohlemüller et al. 2006; Jenerette et al. 2016) and the high cost of training and deploying teams capable of conducting plant community census. A solution to this problem is the use of citizen science, which includes both a strong pedagogical component locally, and simultaneously can produce valuable datasets for a variety of urban forests in a region (Fuccillo et al. 2015). Several studies that measured the accuracy of datasets collected using citizen science consistently indicate that citizen science is an adequate tool for large-scale ecosystem surveys (Aceves-Bueno et al. 2015, 2017). Furthermore, a review by Dickenson et al. (2010) on the use of citizen science for data collection highlights the importance of combining data from separate citizen science programs to adequately monitor trends in ecology across large spatial and temporal scales.

The aim of this study is to investigate factors associated with non-native plant invasions patterns in urban forests, including spatial and land-use attributes of the urban matrix. We provide a citizen science method of data collection and an easily reproducible analysis pipeline to facilitate future studies. Using a template city, we test the hypothesis that urban forest size and relative isolation (i.e., distance to the nearest unfragmented natural forest) guide the incidence of non-native versus native plants species in urban forests. Alternatively, we hypothesize that characteristics of the urban matrix associated with layout and land-use could interfere with the distance and area effects. More specifically, we hypothesize that: (1) larger urban forests closer to the nearest unfragmented forest have greater overall native and non-native species richness as compared to smaller and more distant urban forests, even when accounting for urban matrix characteristics such as population density and surrounding construction events through time; (2) larger urban forests have a smaller ratio of non-native to native species as compared to small ones, even when accounting for urban matrix characteristics such as population density and surrounding construction events through time. Alternatively, urban matrix characteristics may be better predictors of non-native species composition in urban forests and; (3) urban forests that occur closer together show greater similarity in species composition than those farther apart.

# Methods

## Study area

The study area is the city of Sault Ste. Marie, Ontario, Canada. The city lies within the Algoma District bordering the eastern shore of Lake Superior. Sault Ste. Marie has a population of approximately 75,000 (Statistics Canada 2012). The city is part of the Ontario Shield Ecozone, which is characterized by having a large portion of exposed bedrock (Crins et al. 2009). Average daily temperatures during the summer months range from 15.5–17.6 °C with 888.7mm of mean annual precipitation. Located in the center of the Great Lakes-St. Lawrence forest region, the dominant forest types comprise a mixture of both deciduous and coniferous species. Abundant tree species include sugar maple (*Acer saccharum*), yellow birch (*Betula alleghaniensis*), eastern hemlock (*Tsuga canadensis*), white and red pine (*Pinus strobus* and *P. resinosa*), white and black spruce (*Picea glauca* and *P. mariana*), and balsam fir (*Abies balsamea*) (Wake 1997). The city of Sault Ste. Marie is surrounded by relatively undisturbed forested areas which are connected to the vast boreal forests of Canada (Power and Gillis 2006). For the purpose of testing our hypotheses, we consider this to be the 'unfragmented forested area'.

## Urban forest study sites

We obtained the list of study sites by identifying areas zoned by the city of Sault Ste. Marie as parks and recreational areas. We only selected undeveloped and unmanaged forested areas for inclusion in this study and excluded all other heavily managed areas such as sports fields and golf courses. A total of 18 accessible urban forests were identified ranging in size, from 2,200 to 140,5480 m<sup>2</sup> (Table 1, Figure 1). We included in our analysis a forest area that is connected to the unfragmented forest, fragment number 18 (Table 1, Figure 1), and considered it as a control for isolation by distance. We measured urbanization based on the minimum distance to a high traffic road (henceforth "distance to the closest road"), the percentage of land assigned as 'commercial' within a 250 m buffer of the urban forest (henceforth "commercial zoning"), the percentage of land assigned as rural within a 250 m buffer of the urban forest (henceforth "distance to the closest road").

Site number	Area (m <sup>2</sup> )	Perimeter (m)	Distance from Forest (m)
1	2200	271	3480
2	10385	591	4572
3	4274	337	5664
4	6457	431	5299
5	10720	790	4018
6	11963	573	4870
7	11340	467	4284
8	15901	494	3925
9	14062	942	5829
10	17938	708	8496
11	34336	1491	5260
12	11588	616	4669
13	157632	1872	2291
14	315182	2800	1405
15	378112	3514	4196
16	596530	4330	145
17	1405480	6084	7877
18	1028248	5244	0

**Table 1.** Urban forest measurements in Sault Ste. Marie, Ontario. Site number refers to urban forest number of Figure 1.

"rural zoning"), the percentage of land assigned as residential within a 250 m buffer of the urban forest (henceforth "residential zoning"), and the average year of construction within a 250 m buffer of the urban forest (henceforth "average building age") for each urban forest. We obtained the Sault Ste. Marie zoning information from Sault Ste. Marie City Hall (Christopher Bean, GIS Coordinator).

## Sampling design and data collection

We randomly distributed Modified-Whittaker sampling plots within each of the urban forest islands. The Modified-Whittaker sampling design detects greater species richness and is a more convenient sampling method than the Whittaker plot design (Ghorbani et al. 2011). It is a nested vegetation sampling design that allows sampling species richness at multiple scales and to plot species-area relationships. The design nests smaller sub-plots within one main  $1000m^2 (20 \times 50 \text{ m})$  plot. Sub-plots consist of one  $100 \text{ m}^2 (5 \times 20 \text{ m})$  plot placed within the centre of the main plot, two  $10m^2 (2 \times 5 \text{ m})$  plots in opposite corners of the main plot, and ten  $1m^2 (0.5 \times 2 \text{ m})$  plots placed around the border of the main plot (Stohlgren et al. 1995).

A team of 52 citizen scientists went to each of the pre-established plots in the urban forests between July 2 and August 9, 2013 and collected data that enabled the identification of all vascular plants present to species-level. In addition, the citizen scientists counted and provided cover estimates within each of the 1m<sup>2</sup> sub-plots for each species identified. Citizen scientists used general field knowledge as well as personal field guides to help identify each plant to species. For non-native species, we provided a booklet containing descriptions of the most common invasive plants in the area. Specimens that could not be immediately identified to species were collected and tagged.



**Figure 1.** Map (i.e., WGS84 projection) of the 18 urban forests in Sault Ste. Marie, Ontario, Canada, in which this study is based. Both maps contain a representation of each urban forest, site 1 through 18, in green with a 250 m buffer beginning at the edge of the forested area and the unfragmented forest edge in red **A** the year of construction of all the plots with some development is indicated as a blue gradient **B** the population density of each neighborhood is indicated as a gray gradient.

We later identified these specimens with support from the scientists at the Northern Ontario Herbarium, and prepared them to be stored as part of the collection in the Algoma University Herbarium. Cover was estimated using the Braun-Blanquet coverabundance scale (Wikum and Shanholtzer 1978). We then created cumulative plant species lists and cover estimates for each of the 10m<sup>2</sup> and 100m<sup>2</sup> sub-plots and for the main 1000m<sup>2</sup> plot. We then assigned species a native or non-native status according to the PLANTS Database (USDA, NRCS 2018).

#### Geometry calculations

We calculated all geometry features in QGIS (QGIS Development Team 2019). Using Google Earth Pro satellite images, we produced polygons around the urban islands and identified the edge of the larger forest surrounding the city of Sault Ste. Marie (henceforth the "unfragmented" forest). All distances were calculated from a map projected with the coordinate reference system utm16N. We measured area (m<sup>2</sup>) and length (m) of the edge around (henceforth the perimeter) the urban forest polygons. Using the NNJoin pluggin version 3.1.2, we measured the distance between each urban forest from edge to edge and the shortest distance of each urban forest to the "unfragmented" forest line.

To quantify urban landscape use, we added the location of high traffic roads, the year of construction of each plot, and zoning information for plots to our map. We computed the distance between each urban forest and the closest high traffic road. The city zones include rural, environment/natural, mining, park, residential, and commercial land use. To determine impacts of adjacent urban factors on urban forest composition, we used a 250 m buffer, which starts at the edge of each forest and ends 250 m within the urban matrix, around each urban forest. We calculated the percentage designated to each zone in the buffer area, and the average year of construction of plots in the buffer area (Table 1, Figure 1).

## Statistical analysis

We used R version 3.5.1 (R Core Team 2018) to perform all statistical analyses. We produced two types of variables for our analyses. The first type of variables described spatial arrangement of the urban forests and included area, perimeter, and distance to the "unfragmented" forest. The second type of variables describe the urban landscape surrounding the urban forests and included the distance to the closest road, the commercial zoning, the rural zoning, the residential zoning, and average building age. To avoid collinearity among predictor variables, we conducted pair-wise Pearson's correlation tests and kept all variables with a correlation coefficient below 0.7 and above -0.7 (Dorman et al. 2013). When faced with choosing between two variables with a correlation coefficient above 0.7 or below -0.7, we kept the variable that was most ecologically meaningful. Area and perimeter of urban forests were positively correlated (R = 0.96). We kept area in the final model because of the relevance of species-area relationship in ecology (e.g., Connor and McCoy 1979), which was central to our hypotheses. Rural zoning was negatively correlated to residential zoning and positively correlated with area. Again, because of the importance of area in our analysis, we chose to remove rural zoning and keep residential zoning as a variable in the final model.

Since we did not have a priori hypotheses about which variables (i.e., spatial and socio-economic) could better explain species composition, we performed multi-model inference to rank candidate models using Akaike's information criterion corrected for a large number of predictors (AICc). We used this technique to determine which independent variable could better explain the variance in species richness, non-native to native species ratio, non-native species richness, and native species richness. We tested all response variables for normality using the Shapiro-Wilks test. We nested all possible combinations of both the spatial and the urban landscape variables to produce a set of candidate models. To assess variable significance, we calculated the weighted model average of all candidate models within the 95% confidence set of models (sum of model weights > 0.95) (Burnham and Anderson 2004). We considered variables to be relevant when the confidence intervals of the variables in the averaged model did not overlap zero. The estimates of averaged models cannot reliably measure the effect size of a variable (Cade 2015). Instead, we reported the adjusted R-Squared of a model including only the relevant variables and the variance explained by each variable through hierarchical partitioning using hier.part from the hier.part package (Nally and Walsh 2004), which provides a reliable assessment of the strength of the correlation between the dependent and the independent variable (Chevan and Sutherland 1991). We also analyzed the data using 'classical inference', and found that in both analyses similar conclusions were supported by the results (Table 2).

To determine if spatial and landscape variables influenced species composition, we performed a redundancy analysis (rda) and partitioned the variance between spatial and urban landscape variables. We used Hellinger transformed matrices of species composition as the response variables of the rda with the function *rda* from the package *VEGAN* (Oksanen et al. 2018). We included area, area to perimeter ratio, distance to the "unfragmented" forest, distance to roads, all zoning components, and the average year of construction surrounding the urban forest as response variables. None of the variables had a variance inflation factor higher than 3. The significance of the redundancy analysis was tested using *ordiR2step*, from *VEGAN*, with 1000 permutations. The significance of each axis and then each term was assessed similarly using *anova.caa*, from *VEGAN*. We repeated this analysis with only the non-native and the native species to see if the same patterns were found in all the groups.

To test for the relationship between the distance among urban forests and their similarity in community assembly, we used the function *mantel.correlog* from the package *VEGAN*, with Pearson's correlation coefficient and 1000 permutations. We provided the program with a distance matrix of the distance among the urban forests and a similarity matrix composed of species abundance for each urban forest. We calculated the break point between distance classes for the mantel correlogram using the Sturges equation. We repeated this analysis by replacing the similarity matrix with a matrix containing only the non-native species, and then only with the native species to see if different factors affected each group. Both the R script of the analysis and the dataframe of used variables are available as supplementary files (see Supple material 1: 'analysis' for R scrip with the analysis, and Supple material 2: 'data\_csv' for the dataframe of used variables).

Variables	÷.	AICc	AICc		
	Estimate	Lower confidence interval	Upper confidence interval	Estimate	P-value
Kept as part of the model predicting species richness					
Distance from the "unfragmented" forest	0.004	-0.0008	0.0091	0.002	0.542
Kept as part of the model predicting the ratio of native	to exotic sp	ecies			
Residential zoning	0.0036	0.0019	0.005	0.02	0.0001
Average year of construction	0.0077	0.0032	0.0125	0.02	0.239
Distance to the closest road	0	0	0.0001	0.0002	0.0029
Kept as part of the model predicting exotic species rich	ness				
Distance to the closest road	0.0014	0.0004	0.0026	0.002	0.01
Residential zoning	0.1743	0.0751	0.2665	0.17	0.007

Table 2. List of the estimates for the most parsimonious model for each variable.

\* Additional information on the statistical methods is available as supplementary material (Supple material 4: F-statistics).

# Results

# Hypothesis 1: Relationship between urban forest size, distance, and species richness

We found a total of 142 plant species across the urban forests surveyed. Of these species, 36 were non-native and 106 were native. Each urban forest plot contained an average of 16.5 species ( $\pm$ 7.64). On average, 24.19 % ( $\pm$ 21.07) of plants in each plot were non-native, with an average of 4.33 non-native species ( $\pm$ 3.61) and 12.17 native species ( $\pm$ 5.25) per Whitaker plot. The most abundant species were *Maianthemum canadense* (present in 72.22% of plots), *Rubus pubescens* (38.89%), *Fraxinus americana* (33.33%), *Rubus idaeus* (44.44%) and *Acer saccharum* (38.89%). Of these, only *Rubus idaeus* is listed as non-native (see Supple material 3: 'species data').

A total of 64 candidate models were compared to find the best way to predict overall species richness from both spatial and landscape variables. Of the candidate models, 36 were kept as part of the confidence set of the average model, including the Null model (Table 2). The most parsimonious model (with the lowest AICc value) only included distance from the "unfragmented" forest (Estimate (E) = 0.0040) (Figure 2). However, when considering the confidence interval of the effect of distance to the "unfragmented" forest (Confidence interval (CI) = -0.0008, 0.0091), it could not explain species richness. The other variables, including the distance to the closest road (CI = -0.0246, 0.0484), and average building age (CI = -0.0572, 1.6543), were also not considered as being meaningful by the confidence set model average.

# Hypothesis 2: Relationship between urban forest size, distance, urban factors, and native and non-native species richness

We produced three separate groups of candidate models to test which variables affected the ratio of non-native to native species, as well as the non-native and native species, independently. Each response variable produced a total of 64 candidate models. When



**Figure 2.** Each graph represents the linear relationship between urban forest species richness and a variable while controlling for all other variables. Species richness was not related to the area of urban forests (**A**), and it was also not related to distance between the urban forest and the unfragmented forest (**B**).

analyzing the effect of spatial and urban landscape factors on the ratio of non-native to native species, seven models were kept as part of the confidence set of the average model. Area and distance to the unfragmented forested area were not included in the most parsimonious models (Figure 3A, B). The most parsimonious model included residential zoning (E = 0.0036), the average year of construction (E = 0.0077) (Figure 3C), and the distance to the closest road (E = 0.0000) (Figure 3D). The average model from the confidence interval set of models provided similar results; residential zoning (CI = 0.0019, 0.0050), average year of construction (CI = 0.0032, 0.0125), and distance to the closest road (CI = 0.0000, 0.0001) had confidence intervals that showed a positive effect on the non-native to native species ratio. In contrast, commercial zoning (CI = -0.4368, 0.0572), area (CI = -0.0000, 0.0000), and distance from the "unfragmented" forest (CI = -0.0022, 0.0024) did not add to the predictive ability of the model. According to the R-squared adjusted, together, residential zoning, average year of construction, and distance to the closest road explained 69.35% of the variation in the non-native to native species ratio. When we assigned importance to the relevant variables through hierarchical partitioning, we found that residential zoning was the most important variable, with an importance level (I) of 56.48%. However, the distance to the closest road and the average building age were still moderately important (I = 21.88% and 21.63% respectively).

When analyzing the effect of spatial and urban landscape variables on non-native species alone, 18 models were kept as part of the confidence interval set for the model average. The most parsimonious model included a positive effect of the distance to the closest road (E = 0.0014) and residential zoning (E = 0.1743) on the number of non-native species. Similarly, only the distance to the closest road (CI = 0.0004,



**Figure 3.** Linear relationships between the proportion of non-native relative to native species in urban forests and each variable while controlling for variables in the other panels **A** the proportion of non-native to native species was not related to the area of urban islands, calculated in meters squared **B** the proportion of non-native to native species was not related to distance between the urban island and the unfragmented forest, calculated in meters **C** the proportion of non-native to native species increased with newer constructions **D** the proportion of non-native to native species increased with distance from the closest road, calculated in meters.

(0.0026) and residential zoning (CI = 0.0751, 0.2665) had confidence intervals that did not include zero in the model average. Despite the effect of the average year of construction (CI = -0.0662, 0.5207) on the non-native to native species ratio, it did not affect non-native species richness. Together, the distance to the closest road and residential zoning explained 49.43% of the variation in non-native species richness, and residential zoning (I = 72.11%) was more important than the distance to the closest road (I = 27.89%). Commercial zoning (CI = -0.4368, 0.0572), area (CI = -0.0000, 0.0000), and distance from the "unfragmented" forest (CI = -0.0022, 0.0024) did not add to the predictive ability of the model. When analyzing the effect of spatial and urban landscape variables on native species alone, 31 models were kept as part of the confidence interval set for the model average. The most parsimonious model was the null model. The model average had no variable with confidence intervals that did not cross zero. Despite the effect of residential zoning (CI = -0.2936, 0.2477), average year of construction (CI = -0.5293, 1.1767), and distance to the closest road (CI = -0.0018, 0.0040) on the on the non-native to native species ratio, they did not affect native species richness. Commercial zoning (CI = -1.0031, 0.4744), and the spatial variables area (CI = -0.0000, 0.0000) and distance from the "unfragmented" forest (CI = -0.0015,0.0066) were also not considered as meaningful.

We tested the effect of space and urbanization on the overall species composition, the non-native species composition, and the native species composition of the urban forests by partitioning the variance and through model selection of redundancy analysis using permutation tests. The full model could not adequately describe the variance in species composition (F = 0.9821, P = 0.564). Instead, the model that best described the overall species composition was the null model. When only the native species were modeled, we found that none of the variables could explain the patterns of native species composition. Similarly, non-native species composition was not modeled by the set of space and urbanization variables.

# Hypothesis 3: Relationship between proximity of urban forests and species composition

To determine if the proximity of the urban forests influenced their overall, non-native, or native species composition we performed mantel correlogram tests with distance classes calculated using Sturge's equation. According to the mantel correlogram analysis, distance could not predict overall species composition (r = 0.032, P = 0.372). However, urban forests that were in the first distance class, or, in other words, close together, were similar in species composition (P = 0.046) (Figure 4). When separated by native/ non-native status, there was a change in how distance between urban forests affected the similarity in community structure. We found that native species showed no significant spatial correlation in structure (r = 0.001, P = 0.439) (Figure 4), but non-native species had a significant negative linear correlation (r = 0.313, P = 0.001). In fact, urban forests that were closer together were more likely to have similar non-native species (P = 0.001) and those further apart were more likely to be composed by different non-native species (P = 0.006). However, the pattern breaks at the farthest distance class (Figure 4).



**Figure 4.** Mantel's correlogram defaulting to Pearson's correlation coefficient with 1000 permutations. We calculated the distance classes using Sturge's equation. The correlation of distance between urban forests on the similarity in composition of species was tested in four distance classes. Points filled with white represent distances where species composition was not related to distance and solid points represent distances where species. The blue line connecting triangles represent the analysis for native species. The represent the analysis for non-native species.

# Discussion

Our approach using a novel citizen science method of data collection and analysis pipeline enabled identifying whether spatial and/or land-use attributes of the urban matrix were associated with non-native plant occurrence patterns in urban forests. First, it is important to note that the native plants species recorded in our urban forests were consistent with those present in communities of the Great Lakes-St. Lawrence forests of Ontario. The most common native species found in the urban forests, namely Fraxinus americana, Acer saccharum, and Maianthemum canadense, are representatives of the core community of forests in the Great Lakes-St. Lawrence region according to Canada's National Forest Inventory. Similarly, the dominant non-native invasive species reported by the citizen scientists in this study, including Rubus idaeus and Alliaria petiolata, are consistent with reports for this area (e.g., Invasive Species Awareness Program - http://www.invadingspecies.com/). Second, we found that spatial variables did not adequately predict the overall species richness and composition of forest fragments. Both urban forest area and distance to the nearest unfragmented non-urban forest had no effect on plant species richness, suggesting that the urbanization does not always completely isolate the urban forest fragments within the city matrix.

The lack of spatial signal raises the hypotheses that: 1) compared to more densely populated cities, the type of urban development based on detached houses with gardens, which is typical of our model city, may contribute to buffer reproductive isolation among urban forests, and; 2) our urban forests are too recent for ecological effects to have emerged (<100 years). Consistent with this perspective, forest fragments that were adjacent to the relatively less disturbed non-urban forest adjacent to the city showed no sign of hosting more native species relative to the forest fragments imbedded within the city. In contrast, species composition among urban forests responded to residential land-use, construction events, and proximity of roads, suggesting that landscaping and residential planning could be main drivers of non-native species introductions and, eventually, invasion. When considering native and non-native species separately, the proportion of land zoned for residential use surrounding urban forest fragments, recent construction events, measured by the average age of infrastructures near the urban forests, and possibly the distance between these forests and the closest road were factors correlated with an increase in non-native to native species ratio. Additionally, reductions in species composition similarity among urban forests with increasing distance from each other, particularly for non-native species, further indicates that these sites are unlikely to be completely isolated by the urban matrix around them and could be responding to local urbanization factors instead. As such, we conclude that spatial variables, at least in some cases, can be poor predictors of species community richness and non-native species community composition and, instead, we propose that emphasis should be placed on qualities of the urban matrix to determine urban forest non-native community composition.

## The urban matrix and urban forest communities

Our original intent was primarily focused on spatial rather than land use characteristics that meaningfully predict plant community richness and composition. However, the urban matrix variables clearly served to account for factors that, together, could have a larger effect on community composition than the size and spatial arrangement of urban forests. Consequently, we propose that, going forward, studies to predict non-native species community assembly in other urban centers should incorporate characteristics of the urban matrix that are important in urban forest design and management.

While we found compelling evidence that the species richness of urban forests was exclusively driven by parameters relating to the urban matrix, their effect was restricted to the richness of non-native species. These results are congruent with previous studies indicating that human activities (Davis 2009), and particularly the trade of non-native plants in urban areas (Reichard and White 2001; Tartaglia et al 2018), are a major vector for non-native species introductions. The observed increase in non-native species present in the forest fragments near residential areas reinforces the notion that urban forests are likely suitable habitats for these species to escape into (Tartaglia et al. 2018). Additionally, in these residential areas, construction events near urban forests, measured as the average construction year, is a factor positively correlated with non-native species richness. There is a well-established relationship between disturbance events and invasion in the literature (Marvier et al. 2004; Didham et al. 2007; Foxcroft et al. 2011); as such, our results are unsurprising when we think of construction activities as a major potential source of non-native species. However, we found that as the distance from the main roads increased, so did the number of non-native species in forest fragments. We were surprised by these results, as several previous studies have linked road use to non-native invasive species richness (Gelbard and Belnap 2003; Von Der Lippe and Kowarik 2007; Flory and Clay 2009). However, the most common non-native species present in our sites were Rubus idaeus and Alliaria petiolata, which are primarily dispersed by animals; perhaps distance from roads could be associated with greater animal dispersal. Additionally, the incongruence in our results and the literature could be an indicator of historical human development or land-use variables. Again, although these results are not the primary findings of this research, they indicate potentially important characteristics to consider as indicators of urbanization.

This study adds to the body of knowledge on the importance of considering socio-economic factors when analyzing the diversity and species composition of urban landscapes (Hope et al. 2006; González-Moreno et al. 2013; Godefroid and Ricotta 2018; Fan et al. 2019). In fact, ignoring qualities of the urban matrix has been flagged as a limitation of solely using spatial factors; for example, in island biogeography (Laurence 2008). Island Biogeography Theory does not consider the fundamental differences between the ocean, which is largely inhospitable to island species, and the urban matrix which has repeatedly been shown to host sustainable populations of herbaceous species in yards and empty lots (Johnson et al. 2018). Qualities of the urban matrix itself can be used to determine patterns of species composition in urban forests. For example, the proportion of sealed surface in the urban matrix, associated with the degree of urbanization, can be used to predict patterns of species richness in urban forests (Malkinson et al. 2018). Our findings that residential land use (but not commercial use) is associated with an increase in non-native species emphasize the importance of considering variation within the urbanized landscape. Additionally, taking into consideration the urban forest's historical and geographic context when considering patterns of species community assembly is essential. Habitat loss and fragmentation tends to occur in non-random patterns because certain types of habitats, for example areas of low and constant elevation, are better suited to human activity. Thus, these areas are developed more quickly, which attracts further development and loss of habitat, in turn increasing the rate of species loss (Seabloom et al. 2002). As such, it is not completely surprising that overall species patterns cannot be predicted by area and distance relationships alone.

## Application to other urban centers

This study constitutes a first step towards understanding how distance between urban forests and their area affects species composition and patterns of non-native plant invasions. Even though in cities, such as the one used in this study, connectivity between urban forests and proximity to major uninhabited forests may determine the low predictive capacity of spatial attributes alone, that may not be the case in larger urbanized centers, particularly those where habitat fragmentation is high. We know from previous studies that patterns of species composition in urban forests are dependent on both anthropogenic and ecosystem factors at local and regional scales (Ohlemüller et al. 2006; Jenerette et al. 2016). For example, the bioregional context of a city determines the overall species composition of urban forests as well as trends in non-native species composition. Urban forests in the northern regions of North America tend to have less non-native tree species compared to those in the southern regions of the continent, primarily due to the limitations imposed by minimum winter temperatures on trees selected by humans for landscaping purposes (Jenerette et al. 2016). In this context, this study indicates that northern cities, which are not as fragmented or surrounded by fragmented areas as southern cities, can be relatively more protected from biodiversity losses due to non-native plant species introductions. As such, the approach used here can serve as a template to determine larger scale patterns of species composition in future studies, at least across cities in Eastern North America. While this undertaking might require many additional resources and a high degree of coordination, we believe it can be highly effective in the context of invasive species prevention and monitoring. We propose that future studies replicate our methods in other cities to ascertain whether our findings are widely applicable. Additionally, we strongly advocate the use of citizen science as a method of data collection to maximize resources and increase public awareness and knowledge of non-native plants in cities.

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## Supplementary material I

## R scrip of the analysis

Authors: Katherine Duchesneau, Lisa Derickx, Pedro M. Antunes

Data type: statistical data

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Link: https://doi.org/10.3897/neobiota.65.65415.suppl1

# Supplementary material 2

## Dataframe of used variables

Authors: Katherine Duchesneau, Lisa Derickx, Pedro M. Antunes Data type: statistical data

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Link: https://doi.org/10.3897/neobiota.65.65415.suppl2

# Supplementary material 3

## Species data

Authors: Katherine Duchesneau, Lisa Derickx, Pedro M. Antunes

Data type: species data

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# Supplementary material 4

# **F**-statistics

Authors: Katherine Duchesneau, Lisa Derickx, Pedro M. Antunes

Data type: statistical data

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RESEARCH ARTICLE



# Climate change and micro-topography are facilitating the mountain invasion by a non-native perennial plant species

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## Abstract

Mountainous areas and their endemic plant diversity are threatened by global climate change and invasive species. Mountain plant invasions have historically been minimal, however, climate change and increased anthropogenic activity (e.g. roads and vehicles) are amplifying invasion pressure. We assessed plant performance (stem density and fruit production) of the invasive non-native forb Linaria dalmatica along three mountain roads, over an eight-year period (2008–2015) in the Greater Yellowstone Ecosystem (GYE), USA. We evaluated how L. dalmatica performed in response to elevation, changed over time, responded to climate and how the climate of our sites has changed, and compared elevation, climate, micro-topography (slope aspect and angle), and fruit production among sites with differing temporal trends. Linaria dalmatica stem density and fruit production increased with elevation and demonstrated two temporal groups, those populations where stem densities shrank and those that remained stable or grew over time. Stem density demonstrated a hump-shaped response to summer mean temperature, while fruit production decreased with summer mean maximum temperature and showed a hump-shaped response to winter precipitation. Analysis of both short and long-term climate data from our sites, demonstrated that summer temperatures have been increasing and winters getting wetter. The shrinking population group had a lower mean elevation, hotter summer temperatures, drier winters, had plots that differed in slope aspect and angle from the stable/growing group, and produced less fruit. Regional climate projections predict that the observed climate trends will continue, which will likely benefit *L. dalmatica* populations at higher elevations. We conclude that L. dalmatica may persist at lower elevations where it poses little invasive threat, and its invasion into the mountains will continue along roadways, expanding into higher elevations of the GYE.

#### Keywords

Alien Plant Invasion, Climatic Effects, High-elevation Refugia, Linaria dalmatica, Species Range Shift

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# Introduction

Mountainous areas are often regarded as resistant to invasive plant species due to the harsh climate and a low human footprint (Pauchard et al. 2009; Alexander et al. 2016). However, temperatures are rising more rapidly at high elevations (Pepin et al. 2015) and a changing climate, combined with anthropogenic activity, will increase the invasion risk in these areas (Pauchard et al. 2009; McDougall et al. 2018). Non-native plant species abundance is associated with human disturbance and activity (Jauni et al. 2015) and, in mountainous regions, this is commonly roads, their construction and associated vehicular traffic (Pauchard et al. 2009). Seed dispersal by vehicles (Taylor et al. 2012; Rew et al. 2018; Yang et al. 2021) suggests that propagule pressure is likely not limiting along roadsides and the importance of road corridors for dispersal of non-native species has increased attention given to the spread of these species into mountainous areas (Stohlgren et al. 2010; Pollnac et al. 2012; Kalwij et al. 2015; Alexander et al. 2016).

Mountain invasions by non-native plant species generally follow a process called directional ecological filtering (Alexander et al. 2011), where non-native species expand upwards from anthropogenic sources in lowlands, progressively dropping out as the environmental filters associated with increasing elevation become stronger (Alexander et al. 2011; Steyn et al. 2017). Historically, this filtering and the relatively limited anthropogenic activity in mountain and high elevation regions have restricted plant invasions, with non-native plant species richness and abundance decreasing with elevation (Becker et al. 2005; Kalwij et al. 2008; Barni et al. 2012; Seipel et al. 2012; Alexander et al. 2016; Guo et al. 2018). However, global climate change, occurring in mountain and high elevation regions, is weakening the strong climate filter (Pyšek et al. 2011; Carboni et al. 2018), resulting in many species shifting ranges towards higher elevations (Lenoir et al. 2008; Chen et al. 2011). This shift has been demonstrable for non-native plant species (Walther et al. 2002; Kalwij et al. 2015; Koide et al. 2017), which are spreading upwards in elevation twice as fast as native species (Dainese et al. 2017) and anthropogenic propagule sources and roadways are critical for these shifts (Kalwij et al. 2008; Kalwij et al. 2015; Dainese et al. 2017; Lembrechts et al. 2017; Lázaro-Lobo and Ervin 2019). Much of the literature, assessing non-native species along elevation gradients, has used frequency or occurrence data to model species distributions or has described community trends (e.g. species richness) across elevation ranges (Kalwij et al. 2008; Lenoir et al. 2008; Kalwij et al. 2015; Dainese et al. 2017; Koide et al. 2017; Lembrechts et al. 2017; Haider et al. 2018), with few studies describing non-native abundance or reproductive trends over time along mountain elevation gradients (Pauchard et al. 2009; Alexander et al. 2016).

The Greater Yellowstone Ecosystem, USA (GYE) is a mountainous region of high conservation value for its endemic native biodiversity and species of concern. Yellowstone National Park is a conservation area in the region and has management goals of protecting endemic and rare plant species, native biodiversity and limiting and preventing the spread of non-native species (Whipple 2001). Yellowstone National Park and the greater GYE region are attractive tourist destinations accessed almost solely via roadways. Recent research has demonstrated that tourist vehicles in national parks are important seed sources for non-native and fast growing ruderal plant species (Yang et al. 2021), which underscores the risk of roadways facilitating invasions into mountainous conservation areas (Johnston and Pickering 2001; Stohlgren et al. 2010).

The invasive Eurasian perennial forb *Linaria dalmatica* is a species of concern within the region because of its impacts on forage, land values and native plant communities (Robocker 1974; Lacey and Olson 1991). It was first recorded in GYE in 1957 (Whipple 2001), can reproduce vegetatively or via seed and has become widespread throughout the region (Adhikari et al. 2020). Research into the conditions and constraints around the regional invasion of *L. dalmatica* have found contrasting results. *Linaria dalmatica*'s distribution throughout the region has been best described by summer maximum temperatures (McCartney et al. 2019) or February minimum temperatures (Adhikari et al. 2020), while its growth and abundance have been positively associated with precipitation (Blumenthal et al. 2008; Weed and Schwarzländer 2014). Additionally, studies have found no relationship or a negative relationship between *L. dalmatica* and elevation (Pollnac et al. 2014; Rew et al. 2005), but positive associations with steep slopes and southern exposures (Rew et al. 2005; Blumenthal et al. 2012).

The importance of roadways, climate, elevation and topography for non-native species, such as *L. dalmatica*, laid the groundwork for our study, which investigated the temporal and spatial abundance patterns of the *L. dalmatica* along roadways within the mountains of GYE. Specifically, we assessed the environmental constraints shaping *L. dalmatica*'s abundance and propagule pressure along three roads in the mountains of GYE seven times (2008–2013, 2015) over an eight-year period (2008–2015). Our research questions were: 1) Does *L. dalmatica* performance (stem density and fruit production) differ along an elevation gradient? 2) How has *L. dalmatica* stem density changed over the course of the study? Our remaining questions investigated mechanisms driving the observed elevation and temporal trends: 3a) What climate variables best describe *L. dalmatica*'s performance and 3b) how have these climate variables changed over the course of the study and over a longer term (1980–2016)? 4) Do climate (using the variables that best explained stem density and fruit production), elevation, micro-topography (slope angle and aspect) and fruit production, differ between sites where *L. dalmatica* populations are stable/growing and where they are shrinking?

## Materials and methods

## Study area

In 2008, *L. dalmatica* presence was surveyed along three predominately tarred roads near Gardiner, MT, USA (45°01'60"N, 110°42'33"W), one within Yellowstone National Park and the other two just north of the park boundary (Suppl. material 1: Fig. S1A). The road transects proceeded from ca. 1700 m above sea level to the highest elevation extent of the specific roads (2900, 2400, 2200 m). During this initial survey,

all *L. dalmatica* populations within 200 m of the roads were identified and, from the identified populations, six study sites, representing an even spread of elevations along each road, were established. The study sites ranged in elevation between 1736 m and 2316 m. Although our sites were near roads, the community vegetation of our sites was predominantly native and ranged from sagebrush-steppe vegetation at lower elevations to conifer forests at higher elevations. The mean annual temperature (1980–2016) of the study sites was 3.9 °C and the mean annual precipitation was 431 mm. The roads extended beyond the upper elevation range limit of *L. dalmatica* in the region, ensuring dispersal opportunities; see Pollnac et al. (2014) for more site details.

## Field and climate measurements

At each site, ten 1 m<sup>2</sup> monitoring plots were placed randomly in patches of *L. dalmatica*. During the years of the study (2008–2013, 2015), *L. dalmatica* stem density (stems m<sup>-2</sup>) and fruit production (seed capsules m<sup>-2</sup>) were recorded at the end of each growing season (late August) in each monitoring plot. Additionally, in each plot, slope angle was taken with an inclinometer and slope aspect was taken with a compass. We had to remove one site from the study because it was located within a riparian zone that became a construction zone for a period during the project, thereby creating unrepresentative outliers.

Climate data (4 km resolution) used in the analyses were obtained from the University of Idaho's Climate Engine, which uses gridded surface meteorological data, based on PRISM and NLDAS2 (Abatzoglou 2013). Due to proximity, our 18 sites fell into nine climate cells (Suppl. material 1: Fig. S1B), which were collated annually and by season: winter (December–February), spring (March–May), summer (June–August) and autumn (September–November). Autumn climate data used in the analyses were from the year prior to data collection. We downloaded daily minimum, maximum and mean temperatures, as well as daily precipitation and evaluated 24 climate variables: annual variables assessed were mean (°C), mean maximum (°C) and mean minimum (°C) temperatures and precipitation (mm); seasonal variables were the same as the annual variables, additionally, because other studies have determined it to be an important factor for species' growth and distribution (Dainese et al. 2014; Pollnac et al. 2014), the coldest minimum daily temperature (°C) for each season was assessed.

## Data analysis

To address our first question, we first averaged performance data from the ten subplots at each site and created linear mixed effects models by assessing stem density and fruit production across the elevation gradient, with elevation as a fixed effect and site as a random effect to account for repeated measures through time.

To address our second question, we first assessed how stem density changed at each site over time using a linear mixed effects model with stem density as the response variable, year as a fixed effect and plot as a random effect to account for repeated measures through time. In this analysis, we observed sites with shrinking stem densities (n = 9, Suppl. material 1: Fig. S1C, Suppl. material 2: Fig. S2) and sites with stable or growing stem densities through time (n = 8; Suppl. material 1: Fig. S1C, Suppl. material 3: Fig. S3). We then analysed the shrinking/growing population grouped data through time with a linear mixed effects model. In this model, the response was stem density, the fixed effects were shrinking/growing grouping, year (factor) and the interaction between the two and the random effect was site to account for repeated measures through time. Finally, we calculated the yearly stem density estimated marginal means, also known as least-squares mean, for both groups and the Tukey Method was used for post-hoc pairwise comparisons among years for each group and between groups in each year (R-package "emmeans"; Lenth 2020).

To address the first part of our third question, we evaluated the effects of 24 climate variables on L. dalmatica stem density and fruit production. To do this, following similar methodology as previous studies (Barni et al. 2012; Taylor et al. 2014), we first used our 27 environmental variables (24 climate variables, elevation, slope and aspect) to ordinate our sites using principal components analysis (PCA; "rda" in the R-package "vegan"; Oksanen et al. 2019). Linear regression models were then used to assess the strength and direction of the relationship between the PCA axis 1 and each of the environmental variables used to ordinate the plots. We used the climate variables with the strongest relationship with the PCA axis 1 (Table 1) and created linear mixed effects models for both stem density and fruit production ("Imer" in the R-package "ImerTest"; Kuznetsova et al. 2017). For both response variables, additive and interactive climate models were created with uncorrelated climate data (cor. < 0.7; Suppl. material 4: Table S1) as fixed effects. Performance data were averaged across the ten subplots at each site and site was then treated as the random effect to account for repeated measures through time. Road accounted for very little variance as a random effect and models improved without its inclusion. To evaluate for hump-shaped responses, all climate variables were evaluated and compared with and without a second order polynomial transformation. Model creation was followed by manual stepwise backward model selection by removing insignificant variables, from which a final model was chosen by comparing AIC values ( $\Delta$ AIC > 2, indicating a better fit model). If there was no difference ( $\Delta AIC < 2$ ) between final models, the most parsimonious was selected.

For the second part of our third question, we used separate linear mixed effects models for the years of the study (2008–2013, 2015) and over a longer period (1980–2016) using climate variables that were significant in the stem density and fruit production models as responses, year and population group as fixed effects and site as the random effect to account for repeated measures through time.

To address our fourth question, we compared the environment of the shrinking population group with the environment of the stable/growing population group using permutational multivariate analysis of variance with a Euclidean distance ("adonis" in the R-package "vegan"; Oksanen et al. 2019), where the environmental matrix (24 climate variables, elevation, slope and aspect) was the response, population group the predictor variable and year as strata to take into account repeated measures through time. We also used the previously-mentioned principal components analysis to visually compare the differences in environments between the two groups. We then individually compared the climate, elevation, slope angle and aspect, and fruit production, between the population groups. Climate of the population groups was compared in the mixed effects models addressing the second part of question three. Mean elevation of the two groups was compared using a linear mixed effects model, with population group as a fixed effect and site as a random effect. For microsite characteristics without repeated measures (slope angle and aspect), an analysis of variance was performed to compare mean values between our groups. We assessed L. dalmatica's association with the slope aspect for the shrinking and stable/growing groups by comparing the mean absolute value of the degrees from south (180°). Finally, we compared fruit production (response) between population groups (fixed effect) using a linear mixed effects model with site as a random effect. All comparisons between populations were made using pairwise comparisons of the estimated marginal means using the Tukey Method.

The linear mixed effects models' assumptions of normality and homoscedasticity were assessed by visually inspecting model residuals and fruit production and temperature data were log normally transformed. Significant relationships between predictor (fixed effects) and response variables at the P < 0.05 level were calculated from F statistics, based on Satterthwaite's approximations of degrees of freedom. All statistics were performed in the statistical software R, version 3.6.0 (R Core Team 2019) and graphics were produced using the packages "ggplot2" (Wickham 2016), "cowplot" (Wilke 2019) and "ggmap" (Kahle and Wickham 2013).

## Results

## Linaria dalmatica response to elevation

Elevation had the strongest relationship with axis 1 of our principal components analysis, which explained 80% of the variance (Table 1) and both stem density (F = 8.09, Den df = 15, P = 0.012; Fig. 1A) and fruit production (F = 10.77, Den df = 15, P = 0.005; Fig. 1B) increased with elevation.

## Linaria dalmatica's temporal response

In nine of 17 sites, stem density decreased over the eight-year study (shrinking populations), while stem densities at eight sites remained stable (6) or demonstrated a growing (2) trend (Suppl. material 2: Fig. S2). Analysis of this trend on the grouped data (shrinking, stable/growing populations) demonstrated that the stem densities differed between groups (F = 8.62, Den df = 15.0, P = 0.010), that year had an effect on stem

**Table 1.** The primary environmental variables of the study sites. Results from the linear regression models used to assess the strength and direction of the relationship between PCA axis 1 and the environmental variables used to ordinate the 17 study sites.

Variable	Coefficient	SE	Р	$r^2$
Elevation	0.03	< 0.001	< 0.001	0.91
Annual precip.	0.03	0.001	< 0.001	0.82
Summer mean temp	-1.80	0.11	< 0.001	0.71
Winter precip.	0.10	0.006	< 0.001	0.70
Autumn mean max temp.	-1.72	0.10	< 0.001	0.70
Summer mean min temp.	-2.10	0.13	< 0.001	0.68
Annual mean max temp.	-1.80	0.12	< 0.001	0.67
Summer mean max temp.	-1.42	0.09	< 0.001	0.66
Spring precip.	0.06	0.004	< 0.001	0.65
Autumn mean temp.	-2.03	0.14	< 0.001	0.65

Results for the ten environmental variables that were most correlated with PCA axis 1 shown in descending order of importance. 'Precip' represents precipitation, 'temp' represents temperature, 'max' represents maximum and 'min' represents minimum.



**Figure I. A** relationship of *Linaria dalmatica* stem density and **B** fruit production with elevation. Fruit production regression line is back transformed predicted values derived from the mixed effects model.

density (F = 2.80, Den df = 89.0, P = 0.015) and there was an interaction between the population group and year (F = 11.61, Den df = 89.0, P < 0.001). The temporal trend of the shrinking group demonstrated a consistent and substantial stem density decrease over the course of the study (Table 2; Fig. 2). The stable/growing group's increase over the course of the study was consistent, but more gradual and less in magnitude than the decrease of the shrinking group (Table 2; Fig. 2). Comparing stem densities between the shrinking and stable/growing populations through time demonstrated a divergence as the study progressed: in 2008 and 2009, there were no differences between the two groups; however, in each subsequent year, the difference between the two groups increased (Table 3; Fig. 2).

**Table 2.** Assessing *Linaria dalmatica* stem density (stems m<sup>-2</sup>) among years for the shrinking and stable/ growing populations, using estimated marginal means with Tukey pairwise comparisons. Contrast indicates the years being compared, while estimate is the difference between the mean value of the first year compared with the second.

Population group	Contrast	Estimate	SE	df	T. ratio	Р
Shrinking	2008-2012	4.1	1.1	89	3.6	0.01
	2008-2013	5.0	1.1	89	4.4	< 0.001
	2008-2015	6.1	1.1	89	5.3	< 0.001
	2009-2010	3.6	1.2	89	3.1	0.044
	2009-2011	3.7	1.2	89	3.1	0.036
	2009-2012	5.1	1.2	89	4.3	< 0.001
	2009-2013	6.0	1.2	89	5.1	< 0.001
	2009-2015	7.0	1.2	89	6.0	< 0.001
	2010-2015	3.4	1.1	89	3.0	0.054
Stable/growing	2008-2011	-5.8	1.2	89	-4.80	< 0.001
	2008-2012	-4.5	1.2	89	-3.7	0.006
	2008-2013	-4.7	1.2	89	-3.8	0.004
	2008-2015	-4.5	1.2	89	-3.7	0.006

Only shown are those comparisons that met the alpha criterion of 0.05.



**Figure 2.** The estimated marginal means for stem density of those sites with shrinking (dark grey) and stable/growing populations (light grey) across the years of the study (2008–2013, 2015).

## Linaria dalmatica response to climate

Summer mean temperature best described *L. dalmatica*'s stem density response to climate, with stem density demonstrating a hump-shaped response with a peak at 12.5 °C (Table 4; Fig. 3A). *Linaria dalmatica* fruit production decreased with summer maximum temperature (Fig. 3B), but demonstrated a hump-shaped response to winter

Contrast	Year	Estimate	SE	df	T.ratio	Р
Shrinking : growing	2008	-0.2	2.5	22.6	-0.1	0.933
	2009	-2.3	2.5	23.2	-0.9	0.365
	2010	-5.7	2.5	22.6	-2.3	0.034
	2011	-8.7	2.5	22.6	-3.5	0.002
	2012	-8.8	2.5	22.6	-3.5	0.002
	2013	-9.9	2.5	22.6	-4.0	< 0.001
	2015	-10.8	2.5	22.6	-4.3	< 0.001

**Table 3.** Assessing *Linaria dalmatica* stem density (stems m<sup>-2</sup>) between shrinking and stable/growing populations in each year of the study, using estimated marginal means with Tukey pairwise comparison. Estimate is the difference between the mean value of the shrinking group and the stable/growing group.

precipitation, with peak fruit production occurring at ca. 150 mm of winter precipitation (Table 4; Fig. 3C).

Over the years of the study (2008–2013, 2015), summer mean and summer mean maximum temperatures increased (P < 0.001, P < 0.001, respectively). Consistent with this short-term trend, between 1980–2016, summer mean temperatures increased by an average of 0.28 °C per decade (P < 0.001) and summer mean maximum temperatures increased by an average of 0.2 °C per decade (Fig. 4A, B). While winter precipitation demonstrated no temporal trend across the years of our study (P = 0.09), it increased between 1980 and 2016 (P = 0.03), with an average increase of 2 mm per decade (Fig. 4C).

## Environmental comparison between shrinking and stable/growing sites

Our permutational multivariate analysis of variance indicated that environments (climate, elevation, slope angle and aspect) differed between the sites of the shrinking populations and the sites of the stable/growing populations ( $F = 39.0, r^2 = 0.25, P = 0.001$ ). Results of our principal components analysis similarly demonstrated a difference in environments between the two population groups (Fig. 5). There was a substantial trend that summer mean temperatures of the shrinking sites were warmer  $(15.5 \pm 0.71 \text{ °C})$ than the stable/growing sites (13.6  $\pm$  0.66 °C; *P* = 0.06; Fig. 4A). The shrinking sites had significantly warmer summer maximum temperatures ( $24.4 \pm 0.83$  °C) than the stable/growing sites (22.0  $\pm$  0.79 °C; P = 0.05; Fig. 4B) and significantly drier winters  $(60.6 \pm 11.7 \text{ mm})$  than the stable/growing sites  $(98.7 \pm 12.4 \text{ mm}; P = 0.04; \text{ Fig. 4C})$ . Shrinking populations were producing fewer fruit  $(5.02 \pm 1.58)$  than the stable/growing populations (29.05  $\pm$  8.35; F = 17.7, Den df = 15, P < 0.001). Likewise, the sites of the shrinking and stable/growing populations differed in elevation and micro-topography. The mean elevation of the shrinking populations (1900 m) was 183 m lower than the mean elevation of the stable/growing populations (2083 m; F = 5.33, Den df = 15, P = 0.04). Mean slope angle for the shrinking populations (11.2°) was 4.6° less than the mean slope angle of the stable/growing populations (15.9°; F = 16.7, P < 0.001). Across both groups, L. dalmatica was predominantly found on south-facing slopes; however, the mean degrees from south for shrinking populations was 12.2° greater than the stable/growing populations (F = 4.50, P = 0.04).



**Figure 3.** *Linaria dalmatica* stem density response to **A** summer mean temperature and *L. dalmatica* fruit production response to **B** summer mean maximum temperature and **C** winter precipitation. Fruit production regression line is back-transformed predicted values derived from the mixed effects model.

Table 4.	<i>Linaria dalmatica</i> respo	nse to climate.	Results of	the linear	mixed e	ffects mod	els assessin	g L. dal-
<i>matica</i> ster	m density (stems m <sup>-2</sup> ) a	nd fruit produ	iction (see	d capsules	m <sup>-2</sup> ) in	response to	o climate v	ariables.
All respons	se variables underwent	a second order	r polynom	ial transfo	rmation			

Fixed effects							Random effects		
Response	Predictor	Estimate	SE	df	T value	Р	Variance		
							Site	Residual	
Stem density	(Intercept)	9.71	1.22	15.77	7.94	< 0.001	$24.04 \pm 4.90$	$9.49\pm3.08$	
	Poly(su mean)1	-21.33	7.14	103.57	-2.99	0.004			
	Poly(su mean)2	-10.21	5.18	114.05	-1.97	0.051			
Fruit production	(Intercept)	2.55	0.36	11.07	7.03	< 0.001	$2.13 \pm 1.46$	$0.78\pm0.88$	
	Poly(wint precip)1	7.83	1.57	113.99	5.00	< 0.001			
	Poly(wint precip)2	-5.03	1.15	101.80	-4.38	< 0.001			
	Poly(su max)1	-12.74	1.87	108.41	-6.80	< 0.001			
	Poly(su max)2	-3.58	1.39	110.50	-2.57	0.012			

Precip' represents precipitation, 'su mean' represents summer mean temperature, 'su max' represents summer mean maximum temperature.



**Figure 4.** Comparisons of climate trends (1980–2016) between the sites of the shrinking populations (dark grey) and the stable/growing populations (light grey) for **A** summer mean temperature **B** summer mean maximum and **C** winter precipitation. Dashed vertical line denotes the beginning of the study (2008).

# Discussion

High elevation and mountainous regions have historically presented more obstacles to plant invasions than lowlands (Alexander et al. 2011; Alexander et al. 2016) and evidence of upward shifts by non-native species has been limited (Cannone and Pignatti 2014); non-native species richness and abundance declines with elevation (Becker et al. 2005; Kalwij et al. 2008; Seipel et al. 2012; Alexander et al. 2016; Seipel et al. 2016; Guo et al. 2018; Haider et al. 2018). However, recently, non-native species have started to shift higher along elevation gradients in mountainous regions (Becker et al. 2005; Kalwij et al. 2008; Kalwij et al. 2015; Dainese et al. 2017; Koide et al. 2017; Lembrechts et al. 2017). While consistent with these studies, our findings are novel because we observed a temporal increase in abundance of a non-native plant species



**Figure 5.** Principal components analysis among the 17 study sites, based on 27 environmental variables (24 climate variables, elevation, slope angle and aspect). PCA axis 1 explained 80% of the variation and PCA axis 2 explained 13% of the variation. Shrinking sites in each year are dark grey, while stable/growing sites in each year are light grey. Ellipses are standard deviations centred on the mean of each group.

at higher elevations, while previous research modelled shifts in optimal elevations or species distributions using either floristic diversity (i.e. species richness) or occurrence/ frequency data. Abundance and reproduction of non-native species through time has only rarely been assessed across elevation gradients (Pollnac et al. 2014; Seipel et al. 2016) and findings have demonstrated no relationship between elevation and population growth rate (Pollnac et al. 2014) or a decrease in density and propagule production of two non-native species with elevation (Seipel et al. 2016).

Studies have found non-native roadside mountain invasions to be characterised by species with high temperature affinities (Lembrechts et al. 2017) and broad climate ranges (Alexander et al. 2011; Steyn et al. 2017). We demonstrated that, while *L. dalmatica* is observed along the elevation gradient, it has relatively cool and wet climate optima, which are likely driving the density and reproduction patterns observed across our elevation gradient. The directional ecological filtering hypothesis (Alexander et al. 2011) predicts that species are generalists that have migrated upslope due to their broad climate niches and predicts that successful high elevation invaders are nested subsets of a lowland species pool (Alexander et al. 2011; Marini et al. 2013; Steyn et al. 2017). However, by evaluating species traits, McDougall et al. (2018) demonstrated that perennial non-native species comprised a higher percentage of non-native species invading higher elevations than annual non-natives. Our findings demonstrate specific climate optima for *L. dalmatica* that is more abundant at high elevations and less at low elevations, i.e. it is leaning upslope (Breshears et al. 2008). It has been suggested that non-native species that are shifting upwards in elevation are not in equilibrium with their environment and their expansion is not due to climate change, but is due to filling of potential niches (Kalwij et al. 2015). We agree that niche filling is a mechanism driving invasions that are recent and where dispersal or invasion opportunities have previously been limiting. However, we do not believe this is happening with *L. dalmatica* at our sites in the mountains of the Greater Yellowstone Ecosystem. *Linaria dalmatica* was first recorded in GYE in 1957 (Whipple 2001), with historic maps suggesting it was introduced as an ornamental at Mammoth Hot Spring in Yellowstone Park, where it was initially observed spreading mainly along roads (L.J. Rew, unpublished data). Mammoth Hot Springs is geographically close to our sites and we believe with over 60 years of substantial tourist vehicle traffic to facilitate its spread, the species has had ample time and opportunity to achieve equilibrium with the environment along our roads of study.

Biotic characteristics of ecosystems affect non-native plant invasions (Dainese et al. 2014; Richardson and Pyšek 2006) and it is possible that the plant communities of our sites affected the observed differences in abundance. However, we do not believe this is occurring because a previous study, using sites on the same roads as ours, found greater levels of invasion by non-native species in sagebrush habitat compared with conifer forests (Pollnac et al. 2012). Our lower elevation sites were predominantly sagebrush-steppe vegetation, while our higher elevation sites were predominantly conifer forests; therefore, if the biotic characteristics of the sites influenced our results, we would expect higher *L. dalmatica* in the lower elevation sagebrush communities, and not in the higher elevation conifer forests.

The observed upward shifts in elevation by non-native species have so far been closely associated with anthropogenic sources of propagules and roadways (Kalwij et al. 2008; Kalwij et al. 2015; Dainese et al. 2017; Koide et al. 2017; Lembrechts et al. 2017) with surrounding native communities demonstrating higher resistance to invasion (Haider et al. 2010; McDougall et al. 2018). Similarly, intact native communities are currently resistant to *L. dalmatica* invasions (Blumenthal et al. 2012) and the risk of invasion along our elevation gradient decreases as the distance from the road increases (Rew et al. 2005). However, warming and elevated atmospheric CO<sub>2</sub> have been shown to favour non-native over native species (Liu et al. 2017) and the *L. dalmatica* invasion will likely benefit from increases in winter precipitation and atmospheric CO<sub>2</sub> (Blumenthal et al. 2008; Blumenthal et al. 2013). Therefore, while the risk of invasion to interior and intact native communities along our roads is currently low, it will likely increase as the climate warms, winter precipitation increases and atmospheric CO<sub>2</sub> levels continue to rise.

## Linaria dalmatica regional responses

Interestingly, our *L. dalmatica* abundance and reproduction findings are consistent with a recent *L. dalmatica* species distribution model that found elevation and summer maximum temperature to be key drivers of its regional distribution in Montana,

Wyoming and Colorado (McCartney et al. 2019). Similar to our findings, McCartney et al. (2019) concluded that L. dalmatica establishment will likely occur below 2800 m, which is above our highest site. However, McCartney et al. (2019) found a higher summer optimal temperature for L. dalmatica and concluded that its occurrence was associated with warm and dry areas. However, they did not address how their results contrasted with previous findings that found a positive association between L. dalmatica and snowfall and precipitation (Blumenthal et al. 2008; Weed and Schwarzländer 2014), though winter precipitation did remain in their model (McCartney et al. 2019). Our findings were also inconsistent with another recent species distribution model that specifically focused on roadside populations, which found February minimum temperature to be the most important variable for L. dalmatica distribution in the state of Montana (Adhikari et al. 2020). Species distribution models use environmental data and occurrence data to determine relationships and can fail to capture detail, especially with regards to species abundance and reproduction (Greiser et al. 2020 and references therein). This is likely the reason for the discrepancy in results between our study using abundance data in GYE versus the two species distribution models that encompassed the state of Montana (Adhikari et al. 2020) or three states (McCartney et al. 2019). At finer scales, our findings contrasted with a field study conducted along roadsides in the mountains of GYE which found no relationship with *L. dalmatica* population growth rate and elevation (Pollnac et al. 2014). We believe the difference in *L. dalmatica* responses to elevation between our study and Pollnac et al. (2014) stem from study duration. Our study was a long-term field study (seven sampling years over an eight year period) and had enough data to cut through the inter-annual noise to perceive trends with more power than the shorter term study of Pollnac et al. (2014).

Fruit production has not been evaluated in other regional studies and we observed an increase with elevation and one of the most important climate variables was winter precipitation. Initially, this is ecologically perplexing; however, *L. dalmatica* growth and abundance responds positively to snowfall and precipitation (Blumenthal et al. 2008; Weed and Schwarzländer 2014) and we believe our findings indicate ideal growing conditions. Winter precipitation falls as snow in this region, melting in spring to provide ample soil recharge for the plant community. In addition, snow maintains a constant temperature at the soil surface, preventing early season mortality due to freezing events, which could benefit stem survival, thereby increasing seed production. Winter precipitation was not a significant variable for our stem density results because it was correlated with summer mean temperature (> 0.70) and was removed during our model selection process.

## Micro-topography: slope angle and aspect

Mountainous regions provide a strong environmental filter, but are also topographically complex at a local scale (e.g. slope angle and aspect), which alters climate and growing conditions (Geiger et al. 2009; Ackerly et al. 2010; Graae et al. 2012; Lenoir et al.
2013) and can increase germination and shape species distributions (Rew et al. 2005; Blumenthal et al. 2012; Boehm et al. 2021). Micro-topography can provide footholds, or refugia, for non-native species above their ideal climate conditions, thereby facilitating plant invasions into higher elevations (Lembrechts et al. 2018). *Linaria dalmatica*'s preference for steep south-facing slopes is well established (Robocker 1974; Rew et al. 2005; Blumenthal et al. 2012) and our abundant and increasing populations were at higher elevations and on steeper, more directly south-facing slopes. We speculate that microrefugia at higher elevations could be facilitating *L. dalmatica*'s invasion into the mountains of GYE. Simultaneously, our results may provide evidence that microrefugia are facilitating the persistence of *L. dalmatica* at sites where the climate is moving away from ideal conditions. We have demonstrated that *L. dalmatica* has a thermal optimum, which is lower than the warm temperatures of our low elevation sites. While populations at the low elevation sites are shrinking, when compared with growing populations, they are also found on terrain (slope aspect and angle) that reduces solar radiation and temperature, which may be aiding their persistence.

#### Probable response to continued climate change

Our results present evidence that the climate constraints of a mountainous region are weakening with a warming climate (Pyšek et al. 2011; Carboni et al. 2018). In southwest Montana, temperatures have increased by 0.2 °C per decade since the 1950s (Whitlock et al. 2017) and approximately 1.14 °C since *L. dalmatica* was introduced. Consistent with this, we found that between 1980 and 2016, summer mean temperatures of our sites have risen by 0.28 °C per decade, while summer maximum temperatures have risen by 0.2 °C per decade. Under different climate scenarios, this warming pattern is predicted to continue (Whitlock et al. 2017). As mean summer temperature best predicted *L. dalmatica* stem density, with a peak around 12.5 °C and the most abundant and stable populations were found at cooler, higher elevations, the regional warming pattern will likely shift *L. dalmatica*'s optimal climate up in elevation, facilitating *L. dalmatica*'s invasion along the roads of the mountains of GYE.

Our results also suggest that the predicted warming pattern will be detrimental to the *L. dalmatica* trailing edge populations found at lower elevations. The lower elevation *L. dalmatica* populations may be in an extinction lag phase, where extinction (at these locations) has been delayed, but is imminent (Alexander et al. 2018). This delay could be due to the presence of microrefugia and its life history as a perennial plant that can reproduce vegetatively, both which can facilitate the persistence of a species in suboptimal conditions (Eriksson 1996; Cotto et al. 2017). The delay could also be the result of an individual asynchrony with the changing climate (Alexander et al. 2018). However, as the climate continues to change, the lag phase will pass and populations at these sites will likely vanish. This is consistent with Adhikari et al. (2020), who predicted an overall decline of *L. dalmatica* in Montana under climate change.

Interestingly, regional climate predictions suggest precipitation will increase by mid-century (Whitlock et al. 2017). Consistent with this prediction, the winter pre-

cipitation of our sites increased between 1980 and 2016. Fruit production demonstrated a hump-shaped response to winter precipitation, with a relatively high optima. Therefore, if the observed trend continues, *L. dalmatica* will not only become more abundant and stable at higher elevations, it will also produce more seed, which will only further the invasion.

#### Conclusions

In the mountains of the Greater Yellowstone Ecosystem, high elevations, especially on steep south-facing slopes, appear more suitable for *L. dalmatica*, with conditions of many low sites being too warm and dry for the species. Under future climate scenarios, as a warming climate weakens climate restrictions, *L. dalmatica* will likely expand its range along roads into the mountains of GYE, as has been suggested for other non-native species under climate change (Dainese et al. 2017; Guo et al. 2018). At lower elevations, due to microrefugia and its perennial life history, *L. dalmatica* will likely persist for a time, although it is unlikely to spread and will continue to decline, becoming locally extinct with a warming climate.

These findings are important for land managers because they show that high elevation *L. dalmatica* populations are producing seed and potentially acting as source populations. Therefore, we suggest the focus of *L. dalmatica* management be on monitoring current populations at high elevations, surveying for new populations on suitable landscape positions and containing high elevation roadside populations before the invasion expands into interior native communities under a changing climate.

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#### Supplementary material I

# Figure S1. Location of study sites (17) by (A) road, (B) climate cell, and (C) population grouping

Authors: Christian D. Larson, Fredric W. Pollnac, Kaylee Schmitz, Lisa J. Rew Data type: occurrence

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Link: https://doi.org/10.3897/neobiota.65.61673.suppl1

#### Supplementary material 2

# Figure S2. *Linaria dalmatica* stem density (stems m-2) over the course of the study (2008–2013, 2015) at those sites with shrinking populations

Authors: Christian D. Larson, Fredric W. Pollnac, Kaylee Schmitz, Lisa J. Rew

Data type: occurrence

Explanation note: Each site is identified by road and elevation.

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#### Supplementary material 3

# Figure S3. *Linaria dalmatica* stem density (stems m-2) over the course of the study (2008–2013, 2015) at those sites with stable/growing populations

Authors: Christian D. Larson, Fredric W. Pollnac, Kaylee Schmitz, Lisa J. Rew Data type: occurrence

Explanation note: Each site is identified by road and elevation.

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# Supplementary material 4

#### Table S1. Correlation matrix

Authors: Christian D. Larson, Fredric W. Pollnac, Kaylee Schmitz, Lisa J. Rew Data type: statistical data

- Explanation note: Correlation matrix for elevation (m), slope aspect (°) and angle (°), and the temperature (°C) and precipitation (mm) variables from which climate models were built. 'Mean', 'maximum', 'minimum' refer to temperature (°C).
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RESEARCH ARTICLE



# Competitive ability of native and alien plants: effects of residence time and invasion status

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#### Abstract

Competition is commonly thought to underlie the impact of plant invasions. However, competitive effects of aliens and competitive response of natives may also change over time. Indeed, as with time, the novelty of an invader decreases, the accumulated eco-evolutionary experience of resident species may eventually limit invasion success. We aimed to gain insights on whether directional changes in biotic interactions over time or more general differences between natives and aliens, for instance, resulting from an introduction bias, are relevant in determining competitive ability. We conducted a pairwise competition experiment in a target-neighbour design, using 47 Asteraceae species with residence times between 8 years-12,000 years in Germany. We first tested whether there are differences in performance in intraspecific competition amongst invasion status groups, that is casual and established neophytes, archaeophytes or native species. We then evaluated whether competitive response and effects depend on residence time or invasion status. Lastly, we assessed whether competitive effects influence range sizes. We found only limited evidence that native target species tolerate neighbours with longer potential co-existence times better, whereas differences in competitive ability were mostly better explained by invasion status than residence time. Although casual neophytes produced most biomass in intraspecific competition, they had the weakest per-capita competitive effects on natives. Notably, we did not find differences between established neophytes and natives, both of which ranked highest in interspecific competitive ability. This lack of differences might be explained by a biased selection of highly invasive or rare native species in previous studies or because invasion success may result from mechanisms other than interspecific competitive superiority. Accordingly, interspecific per-capita competitive effects did not influence range sizes. Further studies across a broader range of environmental conditions, involving other biotic interactions that indirectly influence plant-plant interactions, may clarify when eco-evolutionary adaptations to new invaders are a relevant mechanism.

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#### Keywords

Asteraceae, biotic interaction, co-existence time, competition experiment, competitive response and effect, eco-evolutionary experience, introduction bias, plant invasion

#### Introduction

Biological invasions are a major driver of global change, posing a threat to native species, communities and ecosystems (Simberloff et al. 2013; Pyšek et al. 2020). For plant invasions, competition is the most commonly invoked mechanism by which alien species impact natives (Levine et al. 2003). Understanding whether alien species benefit from higher interspecific competitive ability and whether the resulting local dominance is linked to large-scale invasion success, is thus crucial for conservation management. According to Parker et al. (1999), an invader's potential impact is the product of abundance, per-capita effect and range size. It has long been suggested that locally-abundant species are generally also the ones that are widespread (Lawton 1993) and that rare species are competitively inferior (Griggs 1940). Accordingly, some studies have found that alien species with higher local interspecific competitive ability may also reach larger range sizes (Milla et al. 2011; Sheppard 2019), although other studies with native species have not consistently found such an effect (e.g. Lloyd et al. 2002). Additionally, performance in intraspecific competition may also be an important determinant of invasion success, given that alien species often dominate in dense monospecific stands.

Given its importance for invasion success and impacts, competition amongst aliens and natives has long been studied (Vilà and Weiner 2004). However, a neglected aspect of competition experiments, so far, is the possibility that competitive effects of aliens and competitive response of natives to aliens might change over longer timescales. As a result of eco-evolutionary changes following the introduction of a new species, the performance and potential impacts of an invader may depend on its residence time; that is, the time since introduction to a new area. In fact, one of the most consistent findings in invasion biology is the importance of residence time, particularly for increasing range sizes (Pyšek and Jarošík 2005; Williamson et al. 2009; Pyšek et al. 2015). Beyond affecting range sizes, residence time also has consequences for other ecological and evolutionary processes affecting the invader and resident plant communities (e.g. Lankau et al. 2009; Lankau 2011; Dostál et al. 2013; Gruntman et al. 2017). With increasing residence time, an invader may adapt to its new environment, such as to new climate conditions (Colautti and Barrett 2013), increasing population growth and spread. Conversely, as the novelty of an invader and of the biotic interactions between invader and resident competitors and antagonists decreases over time, the accumulated eco-evolutionary experience of resident species may eventually limit population growth and spread of invaders (Hawkes 2007; Saul et al. 2013; Sheppard and Schurr 2019; Germain et al. 2020).

As competition for limiting resources may act as a selection pressure, plants with a history of co-existence may have developed niche differentiation or reached a balance in competitive abilities through adaptive evolution (Thorpe et al. 2011). Hence, evolutionary changes in competitive ability with residence time may be important for long-term changes in invasion success. From the invader perspective, competitive effects may increase, for instance, when, due to a release from natural enemies, invaders invest less in defence and instead more in competitive ability (evolution of increased competitive ability hypothesis, Blossey and Nötzold 1995). In contrast, evolutionary responses of native species to invaders are less frequently considered and most evidence involves animals (Strauss et al. 2006). Thereby, high impacts of alien species are frequently explained by the native species being naïve with regards to the novel threat (which, however, can be overcome over time, for example, Bytheway and Banks 2019). The few studies investigating evolutionary adaptation to competition between plants have mostly focused on invasive plants that produce allelochemicals (Callaway et al. 2005; Lankau 2012; Dostál et al. 2013). In this regard, the novel weapons hypothesis posits that invaders may succeed because they have novel weapons to which native species are not adapted (Callaway and Aschehoug 2000). However, native species may also learn to tolerate such novel weapons over time, in which case they should show stronger competitive responses with length of co-existence time. For instance, native grasses growing for 20-30 years with Centaurea maculosa in North America, tolerate the allelochemicals better than native grasses of the same species from uninvaded habitats (Callaway et al. 2005). Overall, a meta-analysis of 53 comparisons from 14 experimental studies showed that experienced natives (plants from populations growing with the invader) had higher levels of growth and reproduction than naïve natives (plants from populations that have not yet experienced invasion) (Oduor 2013). Germain et al. (2020) recently demonstrated that such gained experience in a native annual grass, which led to increased competitive ability, can, in turn, reduce invasion growth rate of an invasive annual grass considerably.

Differences in competitive ability between invader and resident species are not necessarily only the result of such gradual directional changes in competitive interactions. Instead or additionally, there may be *a priori* differences in competitive ability that result from an introduction bias. This means that alien species are not a random sample of all plants of the world. Indeed, alien species tend to have a stronger human association and may, hence, be more adapted to human-modified environmental conditions (Buckley and Catford 2016). Furthermore, species deliberately introduced and grown for ornamental or horticultural purposes (which represent the majority of established alien plants in Europe, Lambdon et al. 2008) tend to be strong competitors or have a ruderal strategy, rather than being tolerant to stressful abiotic conditions (Guo et al. 2019). In contrast to the neophytes, the archaeophytes (alien plants introduced before Columbus' discovery of the Americas in 1492) are ruderal or segetal species, originating from drier and warmer regions of the Fertile Crescent and tend to be adapted to more extensive traditional land use (Chytrý et al. 2008; Zając et al. 2009). Such an introduction bias may thus result in *a priori* differences in competitive ability between native, neophyte and archaeophyte species. Indeed, meta-analyses have shown that invasive neophyte species possess traits that distinguish them from native species and

which may confer higher competitive ability (Ordonez et al. 2010; van Kleunen et al. 2010). Although generally fewer studies considered archaeophytes, they have been shown to differ in traits compared to natives (Knapp and Kühn 2012). Nevertheless, it has also been argued that common natives which are successful in human-disturbed habitats may display similar traits as alien species (Thompson and Davis 2011). The concept of discrete "invasion status" categories (such as casual, established, invasive aliens vs. natives, Blackburn et al. 2011) is often employed in invasion biology and can be helpful in highlighting such differences amongst groups because of an introduction bias. However, invasion status groups are sometimes also used as a proxy for effects of residence time. If these latter mechanisms are relevant, such discrete categories may, however, not fully account for directional changes during the process of biological invasions, whereby such eco-evolutionary mechanisms may be better described by residence time as a continuous gradient (Sheppard and Schurr 2019).

In this study, we aimed to gain insights into whether continuous residence time or categorical invasion status may explain differences in competitive ability between natives and aliens better and whether any such differences have consequences for large-scale invasion success (i.e. if species with higher competitive ability reach larger range sizes). Thereby, we specifically aimed to test if we find directional changes in competitive ability over time, consistent with an increase in biotic resistance of native species to newly-introduced species. Alternatively, we considered whether we rather find evidence for more general differences amongst invasion status groups, resulting either from an introduction bias or other non-directional eco-evolutionary processes. To this end, we conducted a pairwise competition experiment with 47 Asteraceae species along an "alien-native species continuum", including species along a continuous gradient of residence times (Sheppard and Schurr 2019) and also representing the four discrete invasion status categories of casual neophytes, established neophytes, archaeophytes and native species in Germany. Such multi-species experiments, although still rarely used in ecology, are very useful in searching for general patterns and mechanisms (van Kleunen et al. 2014). We studied pairwise competition of five native target species with 47 alien and native neighbour species, varying in residence times from 8 years to 12,000 years. This enabled us to assess whether tolerance (competitive response) of native species to competition from neighbours increases with their length of potential co-existence time, as expected if native species gain eco-evolutionary experience and increase biotic resistance to newly-introduced alien species. Additionally, given the potential importance of intraspecific competition for invasion success, we also assessed performance of each neighbour species.

Overall, we thus address the following research questions: 1) Does performance in intraspecific competition depend on invasion status? Note that in this first question we did not test for effects of residence time since no interspecific interactions were involved (i.e. no variation in potential co-existence times). 2) Does interspecific competitive ability (competitive response of five native targets and competitive effects of all 47 alien and native neighbours) depend on residence time or invasion status? 3) Do interspecific competitive effects (and residence time) influence range size?

## Methods

#### Alien-native species continuum

Our study focused on 47 species of annual Asteraceae along an alien-native species continuum (Sheppard and Schurr 2019) (see Suppl. material 1: Fig. S1), representing species of increasing residence times between 8 years-12,000 years in Germany (including 10 casual neophytes, 15 established neophytes, 15 archaeophytes and 7 natives). We limited our study system to one family so that we could focus on effects of residence time and invasion status within phylogenetically-related species of the same life form (annuals) and habitat (open vegetation, ruderal and segetal habitats). We chose Asteraceae because they are one of the largest plant families globally and highly represented in plant invasions. We chose annual species because this enabled us to not only measure plant performance in terms of biomass, as usually done in competition experiments, but also total seed production, which serves as the best proxy for individual fitness, given that annuals are typically monocarpic. Minimum residence time (MRT), which is used as a proxy for the date of first introduction of a species to a new area, was extracted from various databases (Sheppard and Schurr 2019). Usually, we used seed material collected from a previous experiment conducted at the same location in 2016, where individuals had been growing in low density monoculture mesocosms (Brendel et al. 2021). Thus, we minimised potential performance differences arising from maternal effects. If this seed material was not available, we used the original seed material collected in 2015, whereby five species had been collected from wild populations in Baden-Württemberg and one species originated from a botanic garden (see Suppl. material 1: Table S1, for further details).

### Target-neighbour competition experiment

In March 2017, we set up a pairwise competition experiment at a field station of the University of Hohenheim, Germany (Versuchsstation Heidfeldhof: 48°43'02.1"N, 9°11'03.1"E, 400 m a.s.l.; annual precipitation: 698 mm, mean annual temperature: 8.8 °C). The experiment was set up in a target-neighbour design, whereby we focused on five native Asteraceae as target species (*Crepis pulchra, Hypochaeris glabra, Lapsana communis, Pulicaria vulgaris* and *Senecio viscosus*) and all 47 species as neighbours. The five targets were grown in pots as single individuals, in intraspecific competition and in interspecific competition with all other 46 Asteraceae species, which vary in their MRT and invasion status in Germany. Thereby, we had a single target individual in the middle of the pot, surrounded by multiple individuals of one neighbour species.

The pots were placed in ten rows and each target-neighbour combination was usually replicated four times (for the total number of replicates per species combination, see Suppl. material 1: Table S1). Target-neighbour pots were set up in a fully randomised design. In total, we initially established 904 pots, of which the 20 pots with single targets and the 20 pots with intraspecific competition amongst targets, as well as 804 out of the 864 pots in the target-neighbour design had surviving individuals of both species per pot at harvest. The pots had a volume of 15-litre (0.08 m<sup>2</sup> soil surface area, 33 cm upper diameter, 26 cm lower diameter, 24.5 cm height) and were filled with local field soil. Prior to filling the pots, we added a layer of expanding clay to improve drainage. The pots were constantly watered throughout the growing season with an automatic drip-irrigation system and received a maximum of 1.1 litres per day during the warmest period of the year. We weeded the pots before sowing and regularly throughout the experiment.

Targets and neighbours were established from seeds. As we included such a large number of neighbour species that vary in their growth rates, we aimed for constant strength (in terms of biomass production, rather than number of individuals) of neighbour competition across species. We determined the required number of seeds to be sown for each neighbour species, based on data on the average biomass production and establishment rates from a previous experiment in 2016 (Brendel et al. 2021, see Suppl. material 1: Appendix 1, for further details). In mid-May, 3–5 seeds of the target species were added to the centre of each pot to establish the target individuals. At the same time, the species-specific amount of seeds for the neighbour species was sown around the pot centre. All seeds were covered with a thin layer of sand. We also established additional germination trays in the greenhouse to grow seedlings of the target species as back-up for transplanting (see Suppl. material 1: Appendix 1, for further details).

After setting up the target-neighbour combinations, we noticed that the pots were filled with two different soil types (which was not part of the planned design of the experiment): the field soil originated from two separate deliveries from the same company (Glaser Recycling GmbH, Mönsheim, Germany) and soil analyses indicated that these two deliveries were comparable in soil texture, but differed in nutrient contents. Specifically, we had a nutrient-poor (NO<sub>3</sub><sup>-</sup> 5.48 mg/kg, NH<sub>4</sub><sup>+</sup> 0.27 mg/kg, P 3.06 mg/ kg, with a pH value of 8 and total carbon content 1.58%) and a nutrient-rich (NO<sub>3</sub>-10.19 mg/kg, NH<sup>+</sup> 0.89 mg/kg, P 4.28 mg/kg, with a pH value of 7.7 and total carbon content 2.85%) soil. The soils had a sandy loamy texture (nutrient-poor type: 76% sand, 10% clay and 14% silt; nutrient-rich type: 66% sand, 16% clay and 18% silt). As having two different soil types was not a planned part of the experiment and pots had been allocated in a completely randomised manner, the target-neighbour combinations were spread unevenly between the two soil types: of the surviving pots, most pots were of the nutrient-poor type (647 pots, 16 of which are single targets), with only 21% (177 pots, four of which are single targets) in the nutrient-rich type. One species, Carthamus tinctorius, only occurred in the nutrient-rich soil. Having twice the amount of plant available nitrogen highly influenced biomass production during the season and, hence, we usually analysed data originating from the two soil types separately.

Four weeks after sowing, we assessed the germination success of target and neighbour species. In pots where both the target and neighbour species germinated, we thinned out the target species to one single individual. If the target did not germinate, we transplanted a target species individual from the germination trays. If the neighbour did not germinate, we re-sowed the neighbour species. Pots, in which target and neighbour still did not establish following these measures, were removed from the experiment (see Suppl. material 1: Appendix 1).

#### Data collection

To measure performance of targets and neighbours, we harvested aboveground biomass by mid-October 2017 (at least 17 weeks after sowing) and dried it at 70 °C for 72 hours before weighing. For the neighbours, we also counted the number of established individuals and the total number of flower heads (capitula) per pot as a proxy for reproductive output. For target individuals, reproductive output was measured more precisely, using the total seed mass produced per individual in each pot. The experimental period was long enough to allow seed production of all target species, whereby approximately two thirds of all target individuals produced seeds. To measure seed production, we collected seeds during the experiment from ideally ten intact capitula of each target individual, from which we determined the average seed mass per capitulum. Before harvesting each target individual at the end of the experiment, we counted the number of its vital capitula, to then calculate the total seed mass produced.

In addition to the experimental data, we collected data on range sizes in Germany for each species. We obtained these data from the database of FlorKart, BfN and Net-PhyD Netzwerk Phytodiversität Deutschlands e.V. (www.deutschlandflora.de). This database records species occurrence in each of four quadrants of a grid cell of  $10 \times 6$  arc minutes. We counted the number of occupied quadrants per grid cell for each species. The proportion of occupied cells for each species represents its range size in Germany (Sheppard and Schurr 2019).

#### Data analysis

We analysed all data in R v.4.0 (R Core Team 2020). To address the first question of whether performance in intraspecific competition depends on invasion status, we only focused on neighbour performance, assuming that the single target individual had no relevant competitive effect on its many neighbours. Except in the analysis of establishment success, to ensure this assumption was justified, we excluded all pots where target biomass was larger than neighbour biomass. This occurred in 76 out of 527 cases in the nutrient-poor and in 13 out of 156 in the nutrient-rich soil. We included all pots with surviving neighbours, which were not re-sown a second time. This resulted in 43 species for establishment and 41 species for biomass and number of capitula per pot. We were interested in pot-level performance of neighbours as a better measure for overall invasion success. For this first question of addressing intraspecific performance of neighbour species, we analysed the data of both soil types combined, but included soil type as an explanatory variable. We analysed establishment rates (the number of successfully established neighbour individuals by the end of the experiment compared to number of seeds sown) by means of a generalised linear mixed model with binomial distribution. Square-root-transformed aboveground biomass per pot and (log+1)transformed total number of capitula per pot were analysed with a linear mixed effects model. Fixed effects included the categorical variables invasion status (a factor with four levels: casual neophyte, established neophyte, archaeophyte or native), soil type (a factor with two levels: nutrient-poor and nutrient-rich) and their interaction. Species

was included as a random effect in all models. As the number of seeds sown and seed mass differed between species, we included the log-transformed total seed mass sown per pot as a covariate in all analyses. This covariate should account for initial differences in propagule pressure, as well as potentially higher establishment success for larger seeds and density-dependent thinning for higher seed numbers. We tested significance of the explanatory variables by means of likelihood ratio tests.

For the following questions, we conducted all analyses separately for the two different soil types, because biomass production greatly differed between soil types (see "Competition pressure"). Some of the analyses could only be done for the pots with nutrient-poor soil, for which we had considerably more replicates (see Suppl. material 1: Table S1, for species-specific sample sizes). To address the second question of whether competitive response of targets or competitive effects of neighbours depend on residence time or invasion status, we used two approaches. First, to explore the native targets' tolerance to competition (competitive response), we used a linear mixed effects model to investigate the effect of neighbour biomass on target aboveground biomass and total seed mass, depending on either MRT or invasion status and their interaction with neighbour biomass. Given that the native targets have the longest MRTs, the neighbour MRT here represents the length of potential co-existence times between native targets and neighbours. Aboveground target and neighbour biomass was square-root-transformed in all analyses to meet model assumptions, while the total seed mass was (log+0.001)- and MRT log-transformed. Given that seed production was possible (the individuals survived and all target species produced seeds at least in some instances), we included the zero values in this analysis. We included random effects of target and neighbour species identity. Models using the explanatory variable MRT versus invasion status were compared via the Akaike Information Criterion (AIC), while the significance of the interaction between MRT (or invasion status, respectively) and neighbour biomass was tested with likelihood ratio tests. To ensure that effects are not due to targets being differently affected by transplanting or re-sowing, we conducted a control analysis excluding all pots with transplanted target individuals and/or re-sown neighbours.

Second, we estimated the competitive effect of each neighbour species on the targets. For each neighbour species separately, square-root-transformed neighbour biomass was regressed against square-root-transformed target biomass (across all five target species), including all data from interspecific competition in the nutrient-poor soil (for species-specific sample sizes, see Suppl. material 1: Table S2). The slope of this regression represents the strength of the competitive effect. We then tested in a linear model if the competitive effect of the 46 neighbour species (*C. tinctorius* had to be excluded as it only occurred in nutrient-rich soil) was related to log-transformed MRT or invasion status, again comparing the two models via AIC. To account for the fact that the species-specific regressions varied in their sample sizes and goodness of fit, we weighted the regressions by the inverse of the squared standard error of the slope. However, to highlight the effect of weighing, we below also show the results of non-weighted regression. Due to a lack of data points, competitive effects were not estimated for the nutrient-rich soil type. To address the third research question of whether interspecific competitive effects influence range size when accounting for MRT, we assessed in another weighted regression model whether per-capita competitive effects influence range size. Range size was defined as the logit-transformed proportion of area occupied in Germany and we included log-transformed MRT as a covariate. Per-capita competitive effects were again derived from the slope of the species-specific regressions described above and we used the inverse of the squared standard error of the slope as weights.

#### Data resources

The data underpinning the analysis reported in this paper are deposited in the Dryad Data Repository at https://doi.org/10.5061/dryad.qrfj6q5ff.

#### Results

#### Competition pressure

The number of neighbour individuals in the nutrient-poor soil type ranged between 1 and 22 (median 4, mean 5.1), except for the casual neophyte *Callistephus chinensis*, which reached up to 53 individuals. Nevertheless, this species was within the range of neighbour biomass covered by other species: neighbour biomass ranged from 0.01–15.6 g (median 4.0 g, mean 4.7 g). In the nutrient-rich soil type, between 1 and 33 (for *C. chinensis*, up to 42) neighbour individuals established (median 5, mean 6.0). Neighbour biomass in the nutrient-rich soil ranged from 0.21–68.3 g (median 16.2, mean 19.5 g). Target species were usually negatively affected by competition, whereby their biomass production greatly varied depending on neighbour species (see Suppl. material 1: Fig. S2).

#### Performance in intraspecific competition

Establishment success was not affected by the interaction between soil type and invasion status ( $\chi^2_{3df}$  = 4.44, *P* = 0.218), with the full model only explaining 9.9% (marginal R<sup>2</sup>; 85.7% with random effects, i.e. conditional R<sup>2</sup>) of variance in the data (Fig. 1a). We then tested for significance of the main effects in a reduced model without the interaction. Neither the main effects of soil type ( $\chi^2_{1df}$  = 0.88, *P* = 0.349) nor invasion status ( $\chi^2_{3df}$  = 5.03, *P* = 0.170) were significant, nor even the covariate total seed mass sown.

However, the interaction between soil type and invasion status was highly significant for aboveground biomass (Fig. 1b;  $\chi^2_{3df} = 64.5$ , P < 0.001), with the model explaining 74.1% (87.5% with random effects) of the variance. In separate models for the two soil types, differences in invasion status were highly significant (nutrient-poor soil type:  $\chi^2_{3df} = 31.83$ , P < 0.001, with marginal R<sup>2</sup> of 45.4% and conditional R<sup>2</sup> of 72.7%; nutrient-rich soil type:  $\chi^2_{3df} = 21.43$ , P < 0.001, with marginal R<sup>2</sup> of 38.3% and conditional R<sup>2</sup> of 69.1%). While biomass was generally considerably higher in the nutrient-rich soil, in both soil types, casual neophytes produced most biomass and na-



**Figure 1.** Performance in intraspecific competition at pot-level depending on invasion status and soil type (left bars in darker colours show the nutrient-poor and right bars in lighter colour the nutrient-rich soil type). Performance is shown as **a** establishment success (n = 527/156 in the nutrient-poor/nutrient-rich soil type) **b** square-root-transformed aboveground biomass per pot (n = 451/143) and **c** total number of capitula per pot (shown on a log-scale, n = 449/143). The asterisks show the mean performance per invasion status group and soil type.

tives the least and this difference was more pronounced in nutrient-rich soil (Fig. 1b). Note that these results remained qualitatively similar when removing *C. chinensis* from the analysis, given that this was the casual neophyte that established in considerably higher number of individuals than other species.

For number of capitula, the interaction between soil type and invasion status was not significant (Fig. 1c;  $\chi^2_{3df} = 6.15$ , P = 0.104). The model explained 18.1% (90.2% with random effects) of variance in the data. Testing for significance of the main effects in a reduced model without the interaction showed that more capitula were produced in the nutrient-rich soil ( $\chi^2_{1df} = 266.49$ , P < 0.001), while the effect of invasion status was not significant ( $\chi^2_{3df} = 5.99$ , P = 0.112), although the data suggest that established neophytes and natives tended to produce more capitula compared to casual neophytes and archaeophytes.

#### Competitive response and competitive effects

The models testing the effect of invasion status instead of minimum residence time (MRT) on competitive responses resulted in lower AIC and thus better model performance for both aboveground biomass and total seed mass of native targets in the nutrient-poor soil (Table 1). Thereby, we did not find directional changes in competitive ability in respect to target biomass (i.e. only a marginally significant interaction between MRT and neighbour biomass, Fig. 2a). Instead, we found a significant interaction between neighbour biomass and invasion status (Fig. 2b): native targets generally showed lowest tolerance to competition from natives and established neophytes and were least affected by competition from casual neophytes. For target seed mass, the direction of effects was the same as for biomass (Fig. 2c, d), but the interactions were not significant (Table 1). The control analysis without transplanted targets or re-sown neighbours resulted in qualitatively similar effects (see Suppl. material 1: Fig. S3). In the nutrient-rich soil (Fig. 3), for total seed mass of targets, the model with MRT re-

**Table 1.** Models analysing effects of neighbour biomass on target performance depending on minimum residence time (MRT) or invasion status. For each target performance measure (aboveground biomass and total seed mass, sample sizes for the nutrient-poor and nutrient-rich soil type in parentheses), differences in the Akaike Information Criterion ( $\Delta$ AIC), explained variance (marginal R<sup>2</sup> and, in parentheses, conditional R<sup>2</sup>) and results of likelihood ratio tests (LRT,  $\chi^2$  with degrees of freedom and *P*-values) for the interaction between MRT and neighbour biomass or invasion status and neighbour biomass, are shown. Analyses were done separately for the nutrient-poor and nutrient-rich soil type.

Model	Soil type	Target biomass (n = 615/168)			Target total seed mass (n = 607/167)		
		ΔΑΙΟ	R-squared	LRT	ΔΑΙΟ	R-squared	LRT
MRT	Nutrient-poor	6.12	58.4 (71.6)	$\chi^2_{1df} = 3.34,$	2.75	13.3 (55.6)	$\chi^2_{1df} = 0.98,$
				P = 0.067			P = 0.322
Invasion status	Nutrient-poor	0	59.6 (72.4)	$(\chi^2_{3df} = 15.67,$	0	14.9 (56.0)	$\chi^2_{3df} = 5.62,$
				<i>P</i> = 0.001			P = 0.132
MRT	Nutrient-rich	3.20	47.7 (68.4)	$\chi^2_{1df} = 3.33,$	0	19.1 (47.3)	$\chi^2_{1df} = 2.07,$
				<i>P</i> = 0.068			P = 0.150
Invasion status	Nutrient-rich	0	49.6 (72.3)	$\chi^2_{3df} = 13.04,$	1.07	22.6 (49.6)	$\chi^2_{3df} = 7.24,$
				<i>P</i> = 0.005			<i>P</i> = 0.065



**Figure 2.** Effects of square-root-transformed neighbour biomass on target performance **a**, **b** square-root-transformed aboveground biomass (n = 615) and **c**, **d** total seed mass (shown on a log-scale, n = 607), depending on **a**, **c** minimum residence time (MRT) or **b**, **d** invasion status in the nutrient-poor soil. To illustrate the interaction between continuous MRT and neighbour biomass in (**a**, **c**), a few representative values were chosen.

sulted in lower AIC (Table 1). This model predicted higher tolerance of native targets to competition from neighbour species with higher MRT (in line with the hypothesis of increasing biotic resistance over time, Fig. 3c, although note that the interaction between MRT and neighbour biomass was not significant). Conversely, predictions for the invasion status models were similar to the nutrient-poor soil (Fig. 3b, d).

The regression models to determine competitive effects of the 46 species ranged in R<sup>2</sup> from 0.5–87.7% (mean 41.6%, median 41.9%), with 33 species having *P*values lower than 0.05 (see Suppl. material 1: Table S2). MRT had a negative effect on the slope (competitive effect) of these species-specific regressions ( $F_{1.44}$  = 8.20,



**Figure 3.** Effects of square-root-transformed neighbour biomass on target performance **a**, **b** square-root-transformed aboveground biomass (n = 168) and **c**, **d** total seed mass (shown on a log-scale, n = 167), depending on **a**, **c** minimum residence time (MRT) or **b**, **d** invasion status in the nutrient-rich soil. To illustrate the interaction between continuous MRT and neighbour biomass in (**a**, **c**), a few representative values were chosen.

P = 0.006), explaining 15.7% of variance in the data (whereas, if using non-weighted regression, effect size was weaker and not significant). Hence, species with longer residence times have larger competitive effects (Fig. 4a). However, the invasion status model was better with a difference in AIC ( $\Delta$ AIC) of 12.05, showing a highly significant effect of invasion status ( $F_{3,42} = 9.54$ , P < 0.001, Fig. 4b) and explaining 40.5% of variance in the data (however, without weighting, only half the variance was explained for the invasion status model which was still better by  $\Delta$ AIC = 3.72). Casual neophytes had the weakest, established neophytes and natives the strongest competitive effects (Fig. 4b).



**Figure 4.** Effects of **a** minimum residence time or **b** invasion status on the competitive effect (slope of species-specific regressions). In **a** size of circles shows the square root of the inverse of the standard error of the slope to illustrate weights of data points. The grey dashed line shows the regression line without weighting for comparison. In **b** the asterisks show the mean competitive effect per invasion status group (in black, in grey for the model without weighting).



**Figure 5.** Effects of **a** minimum residence time (MRT) and **b** interspecific competitive effect (slope of species-specific regressions) on range size in Germany (model predictions shown with the other explanatory variable fixed at its mean). Size of circles show **a**, **b** the square root of the inverse of the standard error of the slope to illustrate weights of data points. The grey dashed lines show regression lines without weighting for comparison.

#### Competitive effect and range size

When controlling for the highly significant positive effect of MRT on range size ( $F_{1,43}$  = 69.33, P < 0.001, Fig. 5a), the slope (per-capita interspecific competitive effect,

whereby lower values indicate stronger effects) of the species-specific regressions did not significantly affect range sizes in Germany ( $F_{1,43} = 0.25$ , P = 0.618, Fig. 5b; although, without weighting, there was a marginally significant negative effect, meaning that more competitive species tended to have larger range sizes). The model explained 61.8% of variance in range sizes.

#### Discussion

#### Competitive ability in relation to residence time: is there evidence for directional eco-evolutionary changes in competitive ability?

Our results showed that interspecific competitive ability was generally better explained by categorical invasion status compared to continuous residence time. However, total seed production of targets tended to be less affected by competition with neighbours the longer their potential co-existence times in the nutrient-rich soil. This pattern is consistent with the hypothesis of increasing eco-evolutionary experience (Saul et al. 2013) of targets leading to higher biotic resistance to newly-introduced species over time. Thereby, total seed production greatly varied in our experiment from no seeds at all to a large reproductive output. Given that seed production serves as the best measure of individual fitness for annual species, this variation in response to competition should thus have direct consequences on native population growth and persistence.

Invasions provide a natural experiment with which we can test if plant-plant interactions can drive evolution (Thorpe et al. 2011). Provided that competition is important for fitness and that there is genetic variation in traits related to competitive ability, we may expect adaptation to new competitors over time as a result of natural selection (Lankau 2011). From the perspective of a native plant community (consisting of perennial ruderal grassland species, no Asteraceae), rather than pairwise interactions, Sheppard and Schurr (2019) previously found evidence of increasing biotic resistance to invasion by Asteraceae species of increasing residence times. There are several explanations why, in this study, we only found limited evidence of such an effect. First, context-dependence is clearly important, as highlighted by the contrasting results from the two soil types. Other studies have also found that, in contrast to the competitive effect, competitive response was not consistent with nutrient levels or neighbour identity (Wang et al. 2010). Second, eco-evolutionary changes in competitive ability may simply not be relevant enough in contrast to a priori differences: even under strong selective forces, there are factors constraining adaptation, such as plasticity, spatial heterogeneity in selective forces, gene flow, lack of additive genetic variance, negative genetic correlations or unfavourable demography and population structure (Strauss et al. 2006; Thorpe et al. 2011). Third, instead of, or additionally to, adaptive responses of native plant species to novel competitive interactions, other eco-evolutionary changes in biotic interactions may decrease (or increase) invader performance over time (e.g. Lankau et al. 2009; Dostál et al. 2013; Gruntman et al. 2017; Aldorfová et al. 2020), potentially confounding effects. Finally, there are some limitations with our multi-species approach, because not all study species are necessarily bound to co-occur, meaning that the neighbour's residence time is only a proxy for length of co-existence time with the native target. To minimise this issue, we used a large set of study species, so that individual species pairs should have limited effects on the results and selected species from the same habitats and sourced them in the same region as far as was possible. Nevertheless, even when finding a pattern consistent with our hypothesis. such as in the nutrient-rich soil, we cannot prove that it results from evolutionary adaptation over time. For this, we would need to experimentally manipulate the presence of alien species and compare performance of naïve and experienced native genotypes (Strauss et al. 2006), which considerably limits the temporal scale that can be studied. Hence, to better understand changes in competitive interactions with increasing length of co-existence time, our approach should be combined with studies on population differences within a species (e.g. Germain et al. 2020), whereby to provide conclusive evidence, experiments ideally would be coupled with genetic analyses.

#### Differences in performance and competitive ability depending on invasion status

The finding that invasion status mostly better explained differences in competitive ability compared to residence time might be because of a priori differences between species types due to an introduction bias. Although invasion status can also serve as a proxy for residence time, our results do not support increasing biotic resistance by native species to newly-introduced species as a mechanism, because we did not find directional effects: archaeophytes generally ranked intermediate, with both natives and established neophytes performing best, whereby native targets showed the lowest tolerance to competition from these two groups. Besides introduction bias, these differences might arise from other eco-evolutionary processes that are more specific to certain invasion status groups (e.g. evolution of increased competitive ability hypothesis, Blossey and Nötzold 1995). However, we here did not find support for the often invoked hypothesis that established alien species have higher competitive ability than natives, which may have several reasons. Recent meta-analyses on pairwise plant interactions between natives and aliens found such higher competitive ability only in particular cases which we did not test here: invasive alien species had greater competitive effects on native species than on non-invasive alien species (Kuebbing and Nuñez 2016) and alien plants were better response competitors than native plants (whereas in line with our results, they did not have larger competitive effects than natives, Golivets and Wallin 2018). In addition, as previously suggested, whether aliens and natives differ may also depend on the types of alien and native species comparisons are made (Vilà and Weiner 2004). For instance, in a multi-species experiment, Zhang and van Kleunen (2019) quantified competitive outcomes between 48 pairs of 17 annual natives and established alien neophytes. They found that common aliens (defined as species that are widespread and locally abundant, only two categories: common or rare) were not more competitive than common natives, but only than rare natives.

Furthermore, that aliens themselves are not a uniform category is shown by the substantial differences between casual and established aliens in our study, which may also have important management implications.

Most studies to date did not consider casual neophytes. Indeed, Kuebbing and Nuñez (2016) hypothesised that interaction patterns including casuals may likely differ from established or invasive aliens, but they did not include casuals in their meta-analysis due to the lack of studies available. Casual aliens, as a category, may be more variable in performance than other groups since they have only passed through the transport and introduction stage of the invasion, but not yet through subsequent filters determining establishment and spread (Blackburn et al. 2011). This group includes species that are casual because they did not have enough time to establish, which is indicated by the generally lower residence times (see Suppl. material 1: Fig. S1) and are described by the concept of lag phases (Aikio et al. 2010) or invasion debt (Rouget et al. 2016). This group, however, also includes species that are not successful enough to establish and hence will disappear again with time, representing failed invasions. Thus, it may not be surprising that this group was found to have the weakest per-capita interspecific competitive effects. One species, Bidens ferulifolia, may even facilitate other species, a finding and potential mechanisms that should be further investigated in future studies. The finding that casual neophytes, as a group, produced the highest biomass in intraspecific competition may be because weak competitors generally tolerate intraspecific competition better than interspecific competition (Stoll and Prati 2001). The high biomass production of casual aliens in intraspecific competition also did not lead to higher reproductive output, which could explain why casual neophytes were not yet able to establish self-sustaining populations, even when establishment success did not differ amongst invasion status groups. However, we note that, due to our experimental design that aimed for similar competition pressures amongst species in terms of aboveground biomass production, achieved population densities differed between species due to differing number of seeds sown and additionally differed between replicates due to varying establishment success. Although we corrected for these differences in propagule pressure in our analysis, density-dependence may limit inference of our results. Additionally, since we did not have a true intraspecific competition treatment for the majority of species, the target species may still have had an effect on its neighbours.

Finally, the archaeophytes had the weakest competitive effects after the casual neophytes and an intermediate rank in terms of the native species tolerance to these neighbours. Archaeophytes generally occur in similar habitats to neophytes, but have quite different introduction histories (Chytrý et al. 2008). Their weaker competitive ability may also explain, to some extent, why some archaeophytes are threatened nowadays due to the intensifications in land use. Although they are alien species, their threatened status may be unfortunate, since, as segetal weeds, they are valued for their function in increasing biodiversity in agricultural landscapes (Zajac et al. 2009).

#### Scaling up: relationships between competitive effect and range size

Invasion success may also result from other mechanisms than interspecific competitive superiority. According to the Parker equation, the impact of an invader is the product of abundance, per-capita competitive effects and range sizes (Parker et al. 1999). As several studies have shown before for alien species, residence time strongly influenced range sizes (Pyšek and Jarošík 2005; Williamson et al. 2009; Pyšek et al. 2015). When testing whether large-scale success is associated with local success, we found that percapita interspecific competitive effects did not influence range sizes. Although a couple of studies previously found that more competitive alien species reach larger range sizes (Milla et al. 2011; Sheppard 2019), there are numerous explanations why this might not be the case. Particularly for species with limited residence time, range sizes may depend more on dispersal ability or human introductions. Furthermore, range sizes may also be influenced by herbivory or pollinators (Svenning et al. 2014). We consider it unlikely that variation in the size of potentially suitable habitat may mask effects, as the choice of species was based on similar habitat preference, whereby ruderal and segetal habitats are widespread across Germany. Furthermore, although the species originate from different parts of the world, the species' potentially suitable habitat in Germany, based on climatic niches, is high according to estimates from a previous study (i.e. larger than 90%, calculated using Mahalanobis distances, including all focal species, except four, Sheppard and Schurr 2019).

However, a study on the whole German flora showed that the traits that influence range sizes differed amongst neophytes, archaeophytes and natives (Knapp and Kühn 2012), in which case we may not expect to find a consistent effect across the whole alien-native species continuum considered here. Furthermore, the lack of association between competitive ability and range size may also indicate that our species are r-selected, with fast growth and with it spread in non-competitive environments (Dietz and Edwards 2006). This is a strategy frequently observed in annual species of ruderal habitats, such as we studied here. In fact, for an almost identical set of study species, we previously found evidence for intra- or interspecific selection towards ruderality with increasing residence time: annual Asteraceae species with lower seed mass had higher finite rates of increase and, consistent with selection, species with long residence times had low seed mass (Brendel et al. 2021).

#### Conclusions

Using an alien-native species continuum to investigate pairwise competition amongst 47 Asteraceae species, in this study, we found little evidence of directional changes in competitive ability over long timescales. Large-scale invasion success was also not explained by small-scale competitive ability. Further, despite the well justified reasons to argue that human-mediated invasions differ from natural colonisation (Wilson et al. 2016), we here did not find differences specifically between established neophytes and natives in terms of intra- and interspecific competitive ability. However, there may be other *a priori* differences resulting from an introduction bias and other mechanisms resulting from different eco-evolutionary processes by which the aliens can utilise their novelty to impact on natives that we did not consider here. Further studies across a broader range of environmental conditions, involving other biotic interactions that may indirectly influence plant-plant interactions, may shed light on the contexts in which eco-evolutionary adaptations to new invaders are a relevant mechanism.

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## Supplementary material I

#### Supplementary materials

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Data type: tables, figures

- Explanation note: Appendix 1. Establishment of targets and neighbours. Table S1. The 47 Asteraceae species used in the experiment. Table S2. Linear regressions of species-specific competitive effects of neighbours on the five native target species. Figure S1. Alien-native continuum of the 47 Asteraceae species. Figure S2. Target biomass depending on neighbour species in the nutrient-poor soil. Figure S3. Effects of neighbour biomass on target performance in the control analysis.
- 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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RESEARCH ARTICLE



# Diversity of alien roadside herbs along an elevational gradient in western Mexico

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#### Abstract

Growing numbers of alien plant species threaten natural ecosystems worldwide. Mexico, as a megadiverse country, has lower numbers of alien species than other regions in America. However, there is a lack of information on the factors that determine the diversity patterns of alien species and their relative importance in the vegetation. The diversity of alien roadside herbs was analysed along an elevational gradient in western Mexico, including their relationship with environmental factors. Three hundred and seventeen herbaceous species were found in 37 sampling sites; 10% were aliens. The proportion of alien species in the ruderal herbaceous communities was lower than the average for this vegetation type in the country. Absolute species richness of natives was significantly and positively correlated with elevation. Absolute species richness of aliens was not significantly correlated with elevation, still; higher values were found at lower elevations. Generalised linear models for relative species richness and relative abundance of aliens with elevation had positive estimates near 0, which, though statistically significant, indicated a weak relationship. Other environmental co-factors, such as the distance to roads and highways, climatic variables, and disturbance indicators, were included in a random forest model. No clear correlation patterns were found. This seemingly random distribution of alien herbaceous plants in the region might be caused by the relatively recent introduction of most of the species. They have not yet had time to expand their distribution to their potential maximum. The early stage of the naturalisation process allows us to minimise the further spread of some species with targeted management and prevent them from becoming invasive.

#### **Keywords**

Environmental variables, exotic plant species, ruderal weed species, Sierra de Manantlán

## Introduction

Mexico has far fewer alien species of higher plants, in both relative and absolute numbers, than the USA and Canada, Europe or various South American countries (Espinosa-García et al. 2004b; Villaseñor and Espinosa-García 2004; van Kleunen et al. 2015; Espinosa-García and Villaseñor 2017). A recent survey estimated between 700 and 750 species (Espinosa-García and Villaseñor 2017) which is less than 3% of the flora. Most of them are herbaceous weeds and grasses (Espinosa-García et al. 2004a; Villaseñor and Espinosa-García 2004; Espinosa-García and Villaseñor 2017). They are also, with some exceptions, less successful in terms of the size of their distribution area (Espinosa-García et al. 2004b) or dominance in local weed vegetation.

However, Mexico is a mega-diverse country, and it is risky to generalise results derived from individual local or regional projects (Trejo and Dirzo 2002; CONABIO and SEMADET 2017). Publications on alien plant species in Mexico are scarce, scattered and variable (Espinosa-García and Villaseñor 2017; Ramírez-Albores et al. 2019). Some information is available for the whole country, but most results are restricted to central Mexico or the northern states (Espinosa-García et al. 2004a; Villaseñor and Espinosa-García 2004; Ramírez-Albores et al. 2019). A major effort is needed to study the present-day situation at different scales (Espinosa-García 2009; Espinosa-García and Villaseñor 2017).

Ruderal and roadside vegetation is often the most critical dispersal channel for the initial introduction of alien plants. Roads cross different environmental conditions, such as soils and climate (Kowarik and von der Lippe 2007). After expanding along them, the populations may invade the adjacent vegetation (Kowarik and von der Lippe 2007; Pollnac et al. 2012; Otto et al. 2014; Martínez-De La Cruz et al. 2015; McDougall et al. 2018). Although some studies exist on aliens in roadside vegetation (Otto et al. 2014), this type of vegetation is not well-studied in Mexico (Pollnac et al. 2012; Martínez-De La Cruz et al. 2015; Sánchez-Medrano 2018). In western Mexico, most terrestrial alien plant species grow in highly disturbed areas and roadside vegetation; vegetation without recent disturbances contains only a few aliens (Vázquez-García et al. 1995; Cuevas-Guzmán et al. 2004).

In several studies, low and middle elevations have been shown to host most alien plant species (Pauchard et al. 2009; Alexander et al. 2011; Haider et al. 2018). Temperate regions have their maximum number of alien plants at low elevation, whereas subtropical islands have most aliens at middle elevations (Pauchard et al. 2009). These patterns are not only caused by changes in the abiotic conditions along the elevational gradient and the preadaptation of the alien plants, they also depend on the areas of the primary introduction and highest propagule pressure (Pauchard et al. 2006). In most cases, alien species are introduced at low and middle elevations from where they spread towards higher elevations along roads and other dispersal channels. Anthropogenic disturbances, in general, are also higher at low elevations and foster the establishment of aliens (Pauchard et al. 2006; Alexander et al. 2011; Haider et al. 2018).
Very little literature exists on the distribution of alien plant species along the elevational gradient in tropical or subtropical regions, and the results are highly variable. A study from India found the highest numbers of alien plants at the lowest elevations (Kosaka et al. 2010). An investigation in Ecuador showed a hump-shaped distribution (Sandoya et al. 2017). In contrast, in central Argentina, Paiaro et al. (2010) found that the lowest and highest elevations had more alien plant species than middle elevations. In Mexico, this topic has scarcely been broached (Sánchez-González and López-Mata 2005). One study conducted at elevations from 403 to 1930 m in the north-east of Mexico showed no significant correlation between elevation and alien species number; however, abundance was not analysed (Sánchez-Medrano 2018). This lack of publications and the contradictory results highlight the need to improve the understanding of elevations' influence on the distribution of alien species in warmer regions.

We address the following questions in the paper: 1. How does the invasion level of alien roadside plants vary over an elevation gradient? We expect higher richness at low elevations due to more significant anthropogenic disturbances and shorter distances to seaports and major towns as the initial source of aliens. 2. How does the invasion level relate to other environmental variables representing climate, natural and anthropogenic disturbances? We assume the invasion level to be higher at sites closer to main roads and highways, as well as other anthropogenic disturbances such as fire or grazing intensity. Also, soil compaction should influence the abundance of these plants. We limited our study to herbaceous plants as they are much more common and speciesrich in Mexico, difficult to manage, and with different dynamics than woody species.

# Methods

### Study area

The study area was the Sierra de Manantlán and adjoining regions in south-western Jalisco, Mexico (see Fig. 1). This area includes different vegetation types. Coasts have mangroves, temporarily or permanently flooded, and the xerophytic vegetation of the dunes. Dry and humid tropical forests dominate the coastal plain and lower elevations up to 1700 m (Vázquez-García et al. 1995; CONABIO and SEMADET 2017; Rodríguez-Contreras 2017). Cloud forest occurs at elevations between 700 and 2600 m. Forests of *Quercus* and *Pinus* can be found at higher elevations, with *Abies* in protected valley locations and *Cupressus* on ridges and other exposed areas (Vázquez-García et al. 1995; Rodríguez-Contreras 2017). Much of this area is part of the Sierra de Manantlán Biosphere Reserve, which covers an elevational gradient of 400–2850 m (Vázquez-García et al. 1995).

There are two marked seasons, a dry season with only occasional rains and a rainy season of three to four months from June to September. Precipitation patterns differ depending on the exposure: the southern and south-western slopes are windward and



**Figure 1.** Study area. The biosphere reserve (Reserva de la Biosfera Sierra de Manantlán – RBSM) and its adjoining areas, with the locations of transects and location of the study area in a larger context. Land use and vegetation data from CONABIO (http://www.conabio.gob.mx/informacion/metadata/gis/usv250s6gw.xml?\_httpcache=yes&\_xsl=/db/metadata/xsl/fgdc\_html.xsl&\_indent=no).

humid, and the northern and north-eastern slopes of the Sierra are in the rain shadow and have semi-desertic conditions (Vázquez-García et al. 1995).

The plants' intricate distribution patterns result from the above-mentioned climatic factors combined with the geological and evolutionary history. The estimated percentage of endemic species is relatively high: 1% of the plant species in the Sierra de Manantlán is endemic to the Sierra and 3% to the state of Jalisco (Vázquez-García et al. 1995). Humans have influenced the region with varying intensity since the last Ice Age. In the Biosphere Reserve, recent disturbance has been limited to tree cutting and fire; however, in the surrounding areas we also find urban centres, extensive agricultural and ranching areas, and mining in some locations (Jardel-Peláez 1998; Graf-Montero et al. 2003; Jardel-Peláez et al. 2017).

The study area was located in the administrative region Costa-Sur, a geographic region with less than 3% of the population of the state of Jalisco (Aguirre-Jiménez and Castañeda-Huizar 2017). Threats to the biodiversity of the area range from low to high (Curiel and Garibay-Chávez 2017). Agriculture plays an important role in the regional economy; it is one of the areas with the highest productivity of cattle ranching. In recent decades the mining industry has increased and the coast is influenced by tourism (Jardel-Peláez et al. 2017).



**Figure 2.** Sampling design, showing the transect location in relation to the road or path with the five  $1 \text{ m}^2$  plots.

We worked at sites with ruderal vegetation along an elevational gradient from sea level near the village of La Manzanilla, La Huerta (19°17.78'N, 104°47.67'W) up to elevations just over 2100 m in the Sierra de Manantlán (19°33.82'N, 104°14.95'W). The selection of the locations was preferential and considered the following criteria: elevation, presence of ruderal vegetation, accessibility, and personal security. We searched for appropriate locations from sea level to 2100 m, with an elevational difference of 300 m between them (that is, 0 m, 300 m, 600 m, and so on). We selected ruderal sites with clearly disturbed vegetation near roads, close to abandoned agricultural areas or former pastures (see the map in Fig. 1). We used the concept of Font Quer (1982), which defines the ruderal vegetation as all wild species which form populations in environments altered by human activity, whether or not they are native to the ecosystem; the same definitions were also used by Rzedowski (2006) and Baker (1974).

# Data

The data were obtained from 20 m long transects parallel to roads at a distance of 3 to 5 m from the road, to avoid the modified soil near the roadside. We surveyed four to six transects per elevation level, for a total of 37. Some roads were paved or cobbled, but most were dirt roads. Each transect consisted of five plots of 1 m<sup>2</sup> at meter 1, 5, 10, 15, and 20 of the 20 m transect length (see Fig. 2).

All herbaceous species within the 1 m<sup>2</sup> plot were recorded and identified to species level. We collected several individuals of every unknown morpho-species and at least three individuals of every known species for verification. The vouchers were deposited at the ZEA Herbarium of the University of Guadalajara in Autlán de Navarro. The specimens were identified by consulting specialised literature of the regional flora, identification keys, the Tropicos platform of the Missouri Botanical Garden (www. tropicos.org), and the Herbarium ZEA. One of the co-authors (RCG) is an expert on the regional flora.

We collected 500 specimens, with at least three duplicates each, most of which could be identified to 317 species, and one was a new species not yet published. The remaining specimens could only be determined to genus level, resulting in an additional thirty-five morpho-species. To establish which species were alien, we used the publications of Villaseñor and Espinosa-García (2004) and Espinosa-García and Villaseñor (2017) as reference. The list was checked for synonyms.

We documented information on soil and light conditions in the field, and the coordinates with a Garmin eTrex GPS. The slope was measured using a Haga clinometer. Surface stone cover percentage was estimated in four categories, leaf litter depth measured in cm, and the leaf litter cover estimated in percent. For each plot, we measured compaction of the topsoil in kg per cm<sup>2</sup>, using a pocket penetrometer from Soil Test Inc. A high topsoil density can be an indicator of disturbances such as grazing or movement of vehicles (Woodward 1996; Rab 2004). Tree cover as a proxy for light availability was measured in percent for each plot using a spherical densiometer Model-A (Robert E. Lemmon Forest Densiometers). Fire and grazing intensity were estimated in five categories each, from none to severe influence as suggested by Olvera et al. (1996).

As mentioned above, not all of our transects were near paved roads with continuous traffic. As a proxy for distance to heavier traffic, we used the distance to paved roads and highways, calculated in QGIS Geographic Information System version 3.10.5, an Open-source Geospatial Foundation Project from The Development Team 2018 using the "distance to nearest hub" function. As reference, we used the data for roads and highways from the year 2012 (Atlas de Caminos y Carreteras del Estado de Jalisco 2012) published by the Government of the State of Jalisco (Datos Abiertos. Gobierno de México, https://datos.gob.mx/).

We also considered the mean temperature and annual precipitation, available at a 30 arc s resolution, the so-called "1 km<sup>2</sup> scale", which is equivalent to around 0.86 km<sup>2</sup> at the equator and less towards the poles. The data were downloaded from the Worldclim database (www.worldclim.org), which includes mean values of the period from 1950 to 2000, interpolated from data from different sources (Hijmans et al. 2005). For every transect we calculated the annual mean temperature and the annual precipitation. The variables were obtained using QGIS for the monthly data and R version 4.0.2 (Ihaka and Gentleman 1996) for the annual data. Suppl. material 5: Table S1 compiles the mean values of all captured variables per transect.

### Data analysis

Accumulation curves and the estimated number of species in the pool were calculated for all transects and elevation levels, as quality control, using the functions "specpool" and "specaccum" of the "vegan" package in R (Oksanen et al. 2019). The "specpool" function works with four different equations to extrapolate the species richness in the pool: Chao, bias-corrected Chao, first-order and second-order Jackknife. To discard spatial autocorrelation of species richness patterns, the Moran's I was calculated using the "moran.test" function of the "spdep" package in R (Bivand and Wong 2019).

As a measure of the invasion level, we used the relative alien species richness and relative alien abundance. Both measures consider alien species in relationship with total species (native + alien) richness and abundance of the whole transect (Catford et al. 2012). For the analysis of the relationship of alien invasion level to elevation, generalised linear models were run in R using the "glm" function of the "stats" package (Warton et al. 2016; R Core Team 2020). For total alien and native species richness quasipoisson family was used for the generalised linear model, whereas for the relative alien species richness model the quasibinomial family was selected. For the residuals of all generalised linear models, spatial autocorrelation was tested using the "moran.test" function. Scatter plots and boxplots show the variation of those measures within the elevation levels.

The relation of each environmental variable with alien invasion level, as well as their relative importance and partial dependence, was calculated with the random forest (RF) method. We used the "randomForest" package in R software. RF is a machine learning method based on bagging of classification and regression trees. It is a powerful technique to improve understanding of patterns and processes based on large ecological datasets (Breiman 1996; Liaw and Wiener 2002; Oppel et al. 2009). The program generates a large number of trees, each using a different bootstrap sample of the data, and then searches for the best node split of the tree among a randomly selected subset of predictors. For every bootstrap sample, an unpruned regression tree is grown. The "out-of-bag" (OOB) estimate of error rate is an aggregation of the error rates obtained for each bootstrap iteration (Liaw and Wiener 2002). Results show good performance compared to many other classifiers, and they are robust against overfitting (Breiman 2001; Liaw and Wiener 2002). A pre-selection of variables is not necessary since the RF algorithm works well with continuous and categorical variables and is robust to noise in predictors (Díaz-Uriarte and Alvarez de Andrés 2006; Oppel et al. 2009).

Random forest uses three parameters that have to be defined by the user: the number of trees in the forest, the number of features tried at each node, and the minimum number of data points in each terminal node (Grimm et al. 2008). In some cases, larger numbers of trees obtain more stable results (Díaz-Uriarte and Alvarez de Andrés 2006; Grimm et al. 2008). The number of trees was set to 1000 and the number of variables tried at each node to 4. For the minimum number of data points, we used the default of 5, since higher numbers are only useful for speeding up the calculation time for large datasets. The relative importance of environmental variables was measured as the increase in mean of error (%IncMSE) of a tree in the forest, when the observed values of this variable were randomly permuted in the OOB samples (Genuer et al. 2010). This variable importance measure is also called permutation importance index. This index and the total decrease of node impurity (IncNodePurity) are the measures used by the "varImp" function for RF variable importance (Breiman 2001). Quantification of variable importance is essential to interpret data and understand underlying phenomena in applied problems, and to rank the variables for modelling (Oppel et al. 2009; Genuer et al. 2010).

Random forest models were run for the relative alien species richness and relative alien abundance; both measures were used at transect (5 m<sup>2</sup>) scale. The analysis identified the most important environmental variables (see section "Data"), with the model parameters specified above. Since high correlation between included variables does not affect the results of RF variable importance or model performance in general (Genuer et al. 2010), all environmental variables could be included independently of autocorrelation issues. RF partial dependence plots were printed using the "partialPlot" function to show the partial relation of one variable and the alien species data.

### Results

We identified 317 herbaceous species in the transects, of which 285 were natives and 32 aliens (see Suppl. material 6: Table S2). Thirty-five morpho-species could only be identified to genus level and were not included in further analysis. Based on the "specaccum" analysis our sampling effort covered an estimated 75% to 90% of all roadside species in the area. For each elevation level, the proportion of regional species represented in the transects varied between 64% and 88%, depending on the type of analysis (see the "Data analysis" section). Species richness clustering was highly significant for native species with a z-value of .04 (p-value of .0063; Moran's I index). For alien species, richness appeared to be random (z-value of -.06267 and a non-significant p-value of .525).

Elevation was significantly and positively correlated with the richness of native species, though it only explained a small part of the data (p = .007, Fig. 3A). We found no significance for absolute alien species richness (p = .92), though there were more species at lower elevations (Fig. 3B). The generalised linear model for relative alien species richness and relative abundance showed a decrease with elevation, the tendency was significant but the models' estimates were near zero (relative alien species richness: estimate = -.001 and p-value = .028, 35 degrees of freedom, relative alien abundance: estimate = -.001, p-value = .008, 35 degrees of freedom). In natives both relationships were slightly positive and significant. Spatial autocorrelation could be rejected due to high p-values at the Moran's I test of all generalised linear models. Scatter and boxplots (Fig. 3C,D) show the relative species richness per transect along the elevation gradient and the variation within the elevation levels for alien species.

For relative alien species richness, the most influential variables were (i) the distance to highways, (ii) the elevation and (iii) tree cover, followed by (iv) the annual mean temperature, (v) the slope and (vi) distance to roads (Fig. 4). The relation shown



**Figure 3.** Absolute and relative species richness over elevation in m. Blue lines showing glm predicted values with the 95% confidence interval **A** total species richness of native herbaceous plants over elevation. Glm coefficient = .0003, p-value = .007, 35 degrees of freedom **B** total species richness of alien herbaceous plants over elevation. Glm coefficient = -2.386e-05, p-value = .92, 36 degrees of freedom **C** relative species richness of alien herbaceous plants over the elevation. Glm coefficient = -.0009, p-value = .0276, 35 degrees of freedom **D** relative species richness of alien herbaceous plants, represented by boxplots for each elevation level. They show the median per transect (5 m<sup>2</sup>), third and first quartile and extreme values.

by the partial dependence plots (Fig. 5A–C) of relative species richness to distance to highways was non-linear positive, the relation to elevation was also positive, whereas the relation to tree cover was slightly U-shaped. Both random forest models were significant with low mean squared errors (mean of squared residuals) for relative alien species richness and relative alien abundance. But the variance explained by the models was very low in both cases (see Table 1).

The most important variables for the relative abundance of alien species were (i) the annual mean temperature, (ii) elevation, and (iii) the leaf litter depth, followed by (iv) slope, (v) the distance to highways, and (vi) the tree cover (Suppl. material 1: Fig. S1). The relation of relative alien species abundance to the mean temperature was negative, the relation to elevation was positive and the relation to leaf litter depth was also negative (see Suppl. material 2: Fig. S2, Suppl. material 3: Fig. S3, Suppl. material 4: Fig. S4).



**Figure 4.** Variable importance for relative alien species richness according to the random forest calculations. The figure shows the increase in mean of the error (%IncMSE), also called permutation importance index, on the left side; and the total decrease of node impurity (IncNodePurity) on the right side (distance to highways in m, calculated from open data Jalisco; elevation above sea level in m; tree cover measured per plot; mean annual temperature, obtained from Worldclim data; slope of the transect measured in %; distance to paved roads in m, calculated from open data Jalisco; mean annual precipitation sum, obtained from Worldclim data; soil compaction, measured in kg/m<sup>2</sup>; grazing intensity estimated as an index from 0/none to 5/ severe; leaf litter cover measured in percent of the plot area; stone cover measured in percent of the plot area; leaf litter depth in cm measured in the field; fire intensity estimated as an index from 0/none to 5/severe).



**Figure 5.** Relation of the relative alien species richness to the most important variables. Partial dependence plots for the distance to highways (**A**), the elevation (**B**) and tree cover (**C**).

**Table 1.** Random forest model performance for relative species richness and relative abundance of alien species. It shows the variance in % explained by the model and the mean of squared residuals as measure of model performance is shown.

Model	Variance explained by the model (%)	Mean of squared residuals
Relative species richness	-9.83	.048
Relative abundance	-8.06	.065

# Discussion

The 317 identified species were the equivalent of approximately 12% of the total vascular flora of the Sierra de Manantlán (Vázquez-García et al. 1995). This proportion matches earlier publications on the weed vegetation of Mexico, which show that 10– 12% of the Mexican flora are agrestal or ruderal weeds (Espinosa-García et al. 2004b; Vibrans 2016).

The 32 alien species we identified constitute 10% of the herbaceous flora of the samples. This share is low compared to other regions in the country, where the mean percentage of alien species in weedy vegetation is around 20% (Espinosa-García et al. 2004a, b; Vibrans 2016). The maize field weed vegetation of Puebla and Tlaxcala in central Mexico had 16.4% alien species (Vibrans 1998). A share of 26% of 125 ruderal plants in the urban area of Malinalco in the State of Mexico were aliens (Martínez-De La Cruz et al. 2015). In a study in Mexico City during the 1990s, 37% of 256 ruderal weed species had been introduced from other continents (Vibrans 1998).

The results of the studies are consistent with the remarkably low number of alien plant species that Mexico has in general when seen in a global context, and despite a long history of commercial exchange with Europe, other parts of North America, and Asia (Philippines). The low proportion may be related to the long history of human disturbance and agriculture in the Mesoamerican cultural region. This history promoted the development of native weed communities, which in turn may prevent an excessive invasion of alien plants (Vibrans 1998; Rejmánek et al. 2005; Kühn and Klotz 2007; Vibrans 2016). With further study of the local natural history, we might be able to improve our understanding of this relatively low number of aliens.

Though more alien species were recorded at lower elevations, no statistically clear relationship was found. Spatial autocorrelation could be rejected for all models. However, the tendency partly reflects what the literature has shown for other cases: there are more alien species at lower elevations, mainly due to lower human population densities at high elevations (Alexander et al. 2011; Pollnac et al. 2012; Otto et al. 2014; Haider et al. 2018), but confounding factors may complicate the relationship. In contrast, native species richness of herbaceous plants tends to rise with elevation (Rzedowski and Calderon de Rzedowski 1989), a tendency we also found. Another study in north-eastern Mexico (Sánchez-Medrano 2018) found a similar tendency, but also not significant. The absence of aliens close to the coast might be related to the environmental conditions, where only highly specialised species, such as mangrove species or halophytes can survive (Vázquez-García et al. 1995; CONABIO and SEMA-DET 2017; Rodríguez-Contreras 2017).

Relative alien species richness and abundance patterns were closely related, which means that dominant species react in the same way to environmental variables as less dominant species. The RF models for relative species richness and abundance had a low mean of squared residuals, indicating a good performance. But low (negative) variances explained by the models showed that variances of the species data could not be explained by the environmental variables.

The distance to highways was the most significant variable for relative species richness. Up to a distance of around 1 km the relation was negative. After this it was, unexpectedly, mainly positive and contradicted our hypothesis as well as previous literature (Otto et al. 2014; Haider et al. 2018; McDougall et al. 2018). This variable not only represents the distance to major transport routes where aliens are moved by traffic and can establish first (Kowarik and von der Lippe 2007), but also the distance to human-influenced areas in general. We don't yet have an explanation for this curious phenomenon.

The results from the generalised linear models and the random forest models were contradictory, but in both cases the relations were not very strong. The generalised linear models showed a weak negative relation of the invasion level to elevation. For both random forest models the invasion level was positively related to elevation and negatively to temperature. In other studies alien species richness (not relative species richness) decreased with increasing elevation (Pollnac et al. 2012; Otto et al. 2014; Haider et al. 2018).

McDougall et al. (2018) showed that world-wide species richness patterns vary in function of elevation, depending on certain traits. Perennial species showed an increase with elevation in the roadside vegetation, annual species decreased, but the total of ruderal species richness did not change over elevation (McDougall et al. 2018). In our case, classification into annuals / perennials or ruderal / non-ruderal was not possible because of lack of published information. However, the data suggest intriguing elevational patterns related to biological traits may exist.

The relationship between tree cover and leaf litter depth seems to contradict each other, as both can be considered indicators for disturbance. For the relative species richness model, tree cover was the third most important variable, with a positive relation. For relative abundance leaf litter depth was at the third position, showing a negative relation. However, this contradiction may be explained by some herbaceous alien species requiring sufficient light to grow, although they may germinate below leaf litter (Veldman and Putz 2010).

Our results show that relative alien species richness and abundance were not strongly related to any of the environmental variables included in this study. Different factors may cause this seemingly random distribution. Previous studies show that the influence of environmental variables on the invasion process varies with the ecosystem (Lonsdale 1999; Rejmánek et al. 2005; Richardson and Pyšek 2006; Kühn and Klotz 2007). The number of species and abundance of natives also influences the results, as we are working with relative values. At the 1 m<sup>2</sup> scale higher native species richness can be expected to reduce the probability of finding alien species; at larger scales this relation is inverted (Kühn and Klotz 2007; Pauchard and Shea 2016). As we focused only on roadside vegetation which represent dispersion channels for primary invasion, we suggest that the alien species have not yet concluded the phase of the primary introduction (McDougall et al. 2018).

The conversion from traditional to modern agriculture and urbanisation has caused land-use change on a large scale (Jardel-Peláez 1998; Instituto Nacional de Ecología 2000; Graf-Montero et al. 2003). This change, particularly from agriculture to cattle ranching, has taken place mainly in the last five decades. With this the introduction and propagation of new alien species have risen significantly (Villaseñor and Espinosa-García 2004; Vibrans 2016; Ramirez-Albores et al. 2019). This period is relatively short for the introduced species to establish in their new habitats, and to expand significantly and reach the potential maximum distribution that reflect their biology (Kowarik and von der Lippe 2007; McDougall et al. 2018). Many alien species are known to have a lag-phase during the first decades after introduction, where they remain at low numbers and in few localities before their populations start to increase (Aikio et al. 2010). Therefore, the present-day distribution might represent the lagphase distribution and thus depend mainly on the location and intensity of the initial introduction. To confirm this, further studies should include similar regions in central and western Mexico. Species records from herbarium data could be used to analyse the temporal trends of the invasion, although sampling biases and other potential errors could hamper this (Aikio et al. 2010).

Many African grasses have been planted on large areas as forage for cattle, but only in the last 100 years, and often even more recently (Rzedowski 1990; Williams and Baruch 2000). The intensity of the introduction or propagule pressure is a crucial factor for alien plant distribution patterns. It is difficult to measure and was not included in this study (Richardson and Pyšek 2006). Even though initial introductions of all alien plants are directly linked to human activities (Reichard and White 2001; Kowarik 2005; Kowarik and von der Lippe 2007), the number, distribution, and frequency of repeated introductions can vary considerably.

For eradication success, the size of the distribution area of the alien species and the time since the initial introduction are highly relevant (Coulston 2004; Mack and Lonsdale 2004; West 2004; Gardener et al. 2009; Kettenring and Adams 2011; Pluess et al. 2012). In the case of western Mexico with low total numbers of alien species, which have been introduced recently and not yet expanded, targeted management could make containment possible (Bruce and Johnson 2014). The large number of native weedy plants may help with management. After local eradication of alien species, it is important to re-establish the native flora, and these strong native competitors could be used to initiate appropriate succession (Kettenring and Adams 2011).

# Conclusion

Western Mexico has relatively few alien herbaceous roadside species. They can be found mainly at lower elevations but not directly at the coast. This pattern was expected as introductions occur mainly at lower elevations, where a large part of the land is converted to agricultural land and other uses. Although the relationship was statistically significant, low values of the estimates showed a weak relationship. The result may be related to another finding: the lack of clustering of alien species richness. Both results could be attributed to the fact that most introductions have been relatively recent. The region's alien plant species have probably not yet expanded sufficiently to show clear distribution patterns. For comparison and in order to confirm this assumption, similar studies would have to be conducted in central and western Mexico. It would be interesting to repeat the study in the future and include further plots in the natural adjacent vegetation to see how the situation develops. In the future, and especially if further introductions are not limited to a minimum, and no targeted management is applied, the number and abundance of alien herbaceous plant species will probably increase. This situation can be viewed as an opportunity to prevent the expansion of introduced species and prevent large-scale invasions through management. Identifying the potential invaders and their potential distribution helps to concentrate management strategies on threatened areas.

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# Supplementary material I

# Figure S1

Authors: Isabel Pérez-Postigo

Data type: png image

- Explanation note: Variable importance for relative abundance of alien species according to the random forest calculations. The figure shows the increase in mean of the error (%IncMSE), also called permutation importance index, on the left side; and the total decrease of node impurity (IncNodePurity) on the right side. (distance to highways in m, calculated from open data Jalisco; elevation above sea level in m; tree cover measured per plot; mean annual temperature, obtained from Worldclim data; slope of the transect measured in %; distance to paved roads in m, calculated from open data Jalisco; mean annual precipitation sum, obtained from Worldclim data; soil compaction, measured in kg/m<sup>2</sup>; grazing intensity estimated as an index from 0/none to 5/severe; leaf litter cover measured in percent of the plot area; stone cover measured in percent of the plot area; leaf litter depth in cm measured in the field; fire intensity estimated as an index from 0/none to 5/severe).
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Link: https://doi.org/10.3897/neobiota.65.67192.suppl1

# Supplementary material 2

### Figure S2

Authors: Isabel Pérez-Postigo

Data type: png image

- Explanation note: Relation of the relative abundance of alien species with the most important variables. Partial dependence plot for the annual mean temperature.
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Link: https://doi.org/10.3897/neobiota.65.67192.suppl2

# Supplementary material 3

### Figure S3

Authors: Isabel Pérez-Postigo

Data type: png image

- Explanation note: Relation of the relative abundance of alien species with the most important variables. Partial dependence plot for elevation.
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Link: https://doi.org/10.3897/neobiota.65.67192.suppl3

# Supplementary material 4

#### Figure S4

Authors: Isabel Pérez-Postigo

Data type: png image

- Explanation note: Relation of the relative abundance of alien species with the most important variables. Partial dependence plot for leaf litter depth.
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Link: https://doi.org/10.3897/neobiota.65.67192.suppl4

# Supplementary material 5

# Table S1

Authors: Isabel Pérez-Postigo

Data type: excel table

Explanation note: Mean values of the environmental variables per transect.

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Link: https://doi.org/10.3897/neobiota.65.67192.suppl5

# Supplementary material 6

### Table S2

Authors: Isabel Pérez-Postigo

Data type: excel table

Explanation note: Alien herbaceous species registered in the study area.

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# Revisiting the non-native insect fauna of Greece: Current trends and an updated checklist

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#### Abstract

Alien invasive species not only have adverse environmental impacts, they may also pose socioeconomic and human health threats. The increase in detected non-native (alien) and cryptogenic species, followed by the necessity for up-to-date scientific information on biological invasions, prompted a thorough literature investigation on the non-native entomofauna of Greece. Supplementing previous studies concerning Europe and Greece, a checklist for the non-native insect species of the country is provided, while their number is elevated from 266 to 469. Current trends regarding species richness and taxonomy, deliberate or accidental means of introduction, as well as their native range and trophic preferences are analysed and discussed. This publication illustrates the current state of non-native insects of Greece, highlighting the need for an effective early warning system integrated in national phytosanitary legislation, survey protocols and strategies towards the eradication and mitigation of their detrimental impacts.

#### Keywords

Checklist, insects, invasive species, non-native, trends

# Introduction

Human-mediated species translocations, mostly due to globalisation and international trade, have gradually led to an unprecedented rise in the number of non-native species across the world and which is showing no signs of saturation (Hulme 2009; Seebens et al. 2017). These extraordinary species movements have been indicated as the major cause of a breakdown of "classical" biogeographic regions, through the previously impossible overcoming of geographic dispersal barriers (Capinha et al. 2015). As a consequence, in an era of continuous biotic homogenisation, climate and socioeconomic relationships are foreseen to define biogeography and species assemblages worldwide (McKinney and Lockwood 1999; Capinha et al. 2015).

Invasive alien species (IAS) are responsible for the global loss of biodiversity through, for example, competition with and replacement of native species, pathogen transmission, alteration of food webs and the reduction of habitat complexity (Simberloff et al. 2013; Chinchio et al. 2020). By extension, IAS not only hamper species richness, turnover and distribution, but also they inhibit ecosystem services (Vilà and Hulme 2016). Regarding the socio-economic impact, IAS have been estimated to cost the EU around 12 billion euros annually at least (Kettunen et al. 2009). Additionally, beside the phytosanitary risk, various alien species have been deemed as a public health hazard through the transmission of diseases and allergens, the infliction of venomous bites or the release of poisonous toxins (Schindler et al. 2015; Peyton et al. 2020). These pervasive, contemporary challenges reinforce the necessity for further research on and constant update of the non-native species regarding their introduction, establishment and distribution, but also mitigation of their adverse impact and possible eradication.

The first comprehensive checklist of alien species for Europe was delivered by the DAISIE project (Delivering Alien Invasive Species Inventories for Europe; DAISIE 2009). Under this project, a total of 1306 non-native insects were catalogued (Roques et al. 2009). Since then, the total number of introduced insects in Europe has increased and, according to the available data from the European Alien Species Information Network (EASIN), currently approaches 3000 (of which ca. 1120 are alien within Europe), accounting for approximately one fifth of the total number of alien species in the continent (EASIN 2021).

In Greece, the first national checklist of non-native insect species was published by Avtzis et al. (2017), identifying 266 species. Pursuing the need for up-to-date scientific information on alien species (Tsiamis et al. 2016), this study aims to integrate recent, as well as previously neglected data on the non-native insect species of the country. In this publication, the previously published national checklist (Avtzis et al. 2017) is thoroughly revised, elevating the number of non-native insect species to 469. In addition to complementing the checklist, we discuss the current trends and invasion patterns regarding taxonomy and species richness, status, means of introduction, origin and trophic preferences. The provided checklist and interpreted data are intended to act as a first step towards establishing effective national phytosanitary legislation, a protocol for surveys and a national strategy towards the eradication and mitigation of impacts of non-native insects.

# **Materials and methods**

A thorough literature survey supplementing the previous checklist of non-native insects (Avtzis et al. 2017) led to a large number of peer-reviewed journal articles concerning biological invasions of insects in Greece. Moreover, museum collection data (e.g. Caspers and Creuwels 2020; Fägerström 2020) and records on online databases (e.g. Bahr et al. 2017; Noyes 2019) were integrated into the catalogue. The previous study of Avtzis et al. (2017) focused only on DAISIE records of non-native insects, published until the end of 2014. In this study, additional records, published from January 2015 to 20 January 2021 (e.g. Davranoglou and Koutsoukos 2018; Leivadara et al. 2018; Kalaentzis et al. 2019; Demetriou et al. 2020; Kazilas et al. 2020), as well as previously overlooked publications (e.g. Mroczkowski 1965; Eliopoulos et al. 2002; Koveos et al. 2002; Gatt 2007; Martinou et al. 2011), are also included.

Following the methodological approach of Avtzis et al. (2017), species that have been recorded prior to the 16<sup>th</sup> century were excluded. Additionally, native or migrating species that were included in the previous checklist (Avtzis et al. 2017), were excluded from the present study. Synonymised taxa and biological control agents which failed to be established or with questionable presence were also removed from the checklist, namely: *Rhyzopertha dominica* (Fabricius, 1792) (Panagiotakopulu and Buckland 1991); *Tribolium confusum* (Jacquelin du Val, 1868) (King 2010); *Lasius turcicus* (Santschi, 1921) (Georgiadis, pers. comm.); *Pheidole megacephala* (Fabricius, 1793) and *P. teneriffana* (Forel, 1893) (Salata et al. 2019); *Aphidius colemani* Viereck, 1912, *Dirhinus giffardii* Silvestri, 1913 and *Aphytis lingnanensis* Compere, 1955 (Gerber and Schaffner 2016); *Danaus chrysippus* (Linnaeus, 1758) (Pamperis 2009).

Data on species taxonomy, status, origin, regime and means of introduction (intentional or unintentional) were pooled (Rabitsch 2010; Roques 2010) and statistically analysed in R STUDIO Version 1.2.5042 (R Studio Team 2021). The status of non-native species was catalogued as either alien or cryptogenic, the latter being attributed to species of unknown origin, which are neither demonstrably native nor introduced (Carlton 1996). Origin was interpreted according to biogeographic realms (Udvardy 1975; Snow et al. 1998) and not continents as opposed to Avtzis et al. (2017). Species originating from tropical/subtropical regions were not assigned to certain biogeographic realms due to the vast geographic range that the region covers. Instead, the category was included verbatim as given in Coeur d'Acier et al. (2010), Denux and Zagatti (2010), Pellizzari and Germain (2010), Reynaud (2010) and Sauvard et al. (2010). Species native to multiple biogeographic realms were catalogued accordingly. In agreement with the previous study, Regime consists of four different trophic guilds, namely phytophagous, detritivorous, parasitic and predator species (grouped), while species with unknown trophic preferences were treated as such.

# Results

### Taxonomy and species richness

The complete list of established non-native insect species of Greece is provided (Suppl. material 1). A total of 469 species from 13 orders and 117 families were catalogued (Table 1). Beetles (Coleoptera) are recorded as the richest order accounting for 30.5% of non-native insect species, followed by Hemiptera holding 29.6%. Hymenoptera follow, containing almost one fifth of species (19.4%) while butterflies and moths (Lepidoptera) and flies and allies (Diptera) combined, contain about one tenth of the total number of species (5.8% and 5.1%, respectively).

Compared to the previously recorded 266 non-native species (Avtzis et al. 2017), the current total number is increased by 76%. However, if we take into account the nine aforementioned species that were excluded, new additions increase the total number of non-native insects from the previous checklist by 82% that is, by 212 species. The change in order representation of this study compared to the previous checklist is shown in Fig. 1.

### Origin – status

The origin of alien insects in Greece (excluding cryptogenic species), is summarised in Fig. 2 and Table 2. The Indomalayan, Nearctic and Eastern Palearctic biogeographic realms account each for about one fifth of alien insect species (21%, 21% and 20%, respectively). The Afrotropics contribute 14% of the total species number, followed by the Neotropics responsible for 10%. Only 6% of the country's alien entomofauna originate back to the remote Australian realm, while the lowest percentages were recorded from Western Palearctic and Tropical/Subtropical Regions (4%).

Of all species recorded in this report, 76% account for species alien to Greece, while cryptogenic taxa represent the remaining 24% (112 species) (Table 3).

Order	Families	Species	% Species
Blattodea	2	3	0.6
Coleoptera	28	143	30.5
Dermaptera	2	2	0.4
Diptera	12	24	5.1
Hemiptera	24	139	29.6
Hymenoptera	19	91	19.4
Lepidoptera	13	27	5.8
Mantodea	1	2	0.4
Phthiraptera	5	7	1.5
Psocoptera	6	14	3.0
Siphonaptera	2	3	0.6
Thysanoptera	2	13	2.8
Zygentoma	1	1	0.2
Total	117	469	

Table 1. Family and species richness of the non-native insect species of Greece.



**Figure 1.** Number of non-native insect species per order. The number of species as in Avtzis et al. (2017) is coloured blue, while additions from this study are coloured red.

Order					Origin			
	Western Palearctic	Eastern Palearctic	Afrotropical	Indomalayan	Australian	Nearctic	Neotropical	Tropical/ Subtropical
Blattodea	0	0	2	0	0	0	0	0
Coleoptera	6	16	15	26	9	13	14	10
Dermaptera	0	1	0	1	0	0	0	0
Diptera	0	0	3	2	1	8	2	0
Hemiptera	6	31	9	23	6	33	13	5
Hymenoptera	2	20	20	20	7	23	6	0
Lepidoptera	1	5	3	6	2	2	3	0
Mantodea	1	1	1	0	0	0	0	0
Phthiraptera	0	0	0	0	0	1	0	0
Psocoptera	1	2	0	2	0	1	0	0
Siphonaptera	0	1	1	0	0	0	0	0
Thysanoptera	0	1	2	3	1	2	3	1
Zygentoma	0	0	0	0	0	0	0	0
Total	17	78	56	83	26	83	41	16

Table 2. Origin of non-native insect orders of Greece, excluding cryptogenic species.



Figure 2. Origin of insect species alien to Greece.

Table 3. Sta	atus of non-n	ative insect or	ders of Greece.
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Order	Order Status		
	Alien	Cryptogenic	
Blattodea	2	1	
Coleoptera	98	45	
Dermaptera	1	1	
Diptera	16	8	
Hemiptera	119	20	
Hymenoptera	82	9	
Lepidoptera	18	9	
Mantodea	2	0	
Phthiraptera	1	6	
Psocoptera	4	10	
Siphonaptera	2	1	
Thysanoptera	12	1	
Zygentoma	0	1	
Total	357	112	
Total (%)	76	24	

# Means of introduction (intentional or unintentional)

The vast majority of non-native insects are shown to have been accidentally introduced to the country (89% – 415 species). Intentional introduction occurred at a percentage of only 12%, concerning the deliberate translocation of species for biological control, mainly Hymenoptera (47 species), followed by Coleoptera (6 species) and only one species of Hemiptera.

### Regime

The analysed data suggest that one in two of the presented non-native insects are phytophagous (50%), followed by parasitic/predacious taxa (28%) and detritivorous insects (21%) (Table 4).

# Discussion

### Taxonomy and species richness

Contrary to Avtzis et al. (2017), where Hemiptera dominated other orders in species richness, new additions render Coleoptera as the order holding the highest number of non-native insect species (Fig. 1). Order representation coincides with Roques (2010), as Coleoptera, Hemiptera and Hymenoptera (in descending order) contribute the highest numbers of non-native insects to Europe, as well as to Greece. These orders account for nearly 80% of total non-native insect species in Greece. From a further analysis of some of the most species-rich families within these orders, some general trends can be deducted.

The majority of non-native hymenopteran species (60%) belongs to the superfamily Chalcidoidea that includes some of the most commonly used agents of biological control that are intentionally released, for example, *Coccophagoides utilis* Doutt, 1966 (Argyriou and Kourmadas 1979), *Metaphycus lounsburyi* (Howard, 1898) (Macropodi 1985) and *Semielacher petiolata* (Girault, 1915) (Michelakis and Vacante 1997).

In relation to Hemiptera, the superfamily Coccoidea holds almost half of the nonnative true bugs (41%), followed by the family Aphididae (34%). Both taxa include small, phytophagous insects transported through international commerce of infested

Order		egime		
	phytophagous	detritivorous	parasitic/predator	unknown
Blattodea	0	3	0	0
Coleoptera	58	69	26	2
Dermaptera	0	2	2	0
Diptera	11	7	6	0
Hemiptera	134	0	6	0
Hymenoptera	10	0	81	0
Lepidoptera	19	8	0	0
Mantodea	0	0	2	0
Phthiraptera	0	0	7	0
Psocoptera	0	13	0	1
Siphonaptera	0	0	3	0
Thysanoptera	12	0	1	0
Zygentoma	0	1	0	0
Total	244	103	134	3
Total (%)	50	21	28	1

Table 4. Regime of non-native insect orders of Greece.

plant material (Rabitsch 2010), that are frequently responsible for extensive damage in crops and agricultural plants, for example, *Aphis illinoisensis* Shimer, 1866 (Aggelakis et al. 2005) and *Parasaissetia nigra* (Nietner, 1861) (Tsagkarakis et al. 2016).

Finally, regarding Coleoptera, their numbers extend to a great variety of families; yet, Chrysomelidae, Dermestidae and Nitidulidae seem to hold the largest number of species accounting for almost one third (30.8%) of non-native beetles. These families include some notorious pests that feed on sap, debris, plant material and stored products. For example, non-native species of the genera *Carpophilus* and *Epuraea* (Nitidulidae) have been proven to be major pests of dry, rotten and ripe fruits (Jelínek et al. 2016). Carpet beetles (Dermestidae), such as *Anthrenus coloratus* Reitter, 1881 and *Reesa vespulae* (Milliron, 1939), have been associated with the degradation of leather, animal and wool products, as well as the destruction of museum collections, negatively affecting the preservation of historical cultural heritage (Kovalenko 2018; Tsvetanov and Háva 2020). Additionally, many alien seed-beetles (Chrysomelidae: Bruchinae) have been recorded as pests of a vast spectrum of indigenous and exotic plants (Yus-Ramos et al. 2014). On the contrary, families with wood-boring and saproxylic species (e.g. Cerambycidae, Buprestidae) or ground-dwelling species (e.g. Carabidae) are significantly less represented.

Since the previous study, non-native representatives of three additional insect orders have been recorded in Greece, namely Dermaptera, Mantodea and Zygentoma. The cryptogenic silverfish *Ctenolepisma longicaudata* Escherich, 1905 has been known as a household pest commonly found in attics, cupboards and drawers, first being reported in the country by Mendes (1980). Mantodea are represented in Greece by two alien species, the recently reported giant African mantis *Sphodromantis viridis* Forsskål, 1775 and the giant Asian mantis *Hierodula tenuidentata* Saussure, 1869 (Romanowski et al. 2019; Battiston et al. 2020). Although mantids are considered highly predacious, the ecological impacts of these species on native biodiversity have yet to be studied. Concerning earwigs (order Dermaptera), two species have recently been found in the country, namely *Euborellia femoralis* (Dohrn, 1863), an alien species collected in mainland Greece and the island of Rhodes (Kalaentzis et al., in press) and *Forficula lucasi* Dohrn, 1865, a cryptogenic species collected from the island of Lesvos (Zafeiriou et al. 2021).

### Origin – status

In terms of geographic origin, the largest proportion (41%) of alien insect species originates from Asia (i.e. Eastern Palearctic and Indomalaya). This may arise from the increased import of goods from Asian countries (e.g. China, India and Iraq). A recent example of an Asian invader has been the alarming case of *Xylotrechus chinensis* (Chevrolat, 1852). The species was first intercepted from the island of Crete in 2017, causing the increased mortality of several mulberry trees (Leivadara et al. 2018). No more than two years after its initial record, *X. chinensis* had spread to the capital city of Athens, initiating an eradication effort that has resulted, until now, in the trimming and logging of approximately 1300 infested trees. A somewhat smaller, but equally important percentage of alien insect species, originates from the New World (Nearctic and Neotropics; 31%). The Nearctic zoogeographical realm seems to be the primary source of

non-native Hemiptera, Hymenoptera and Diptera, while Indomalaya contributes to the majority of alien Coleoptera and Lepidoptera.

For many non-native insects, it is generally unknown whether their introduction to Greece is the result of a primary introduction event from their area of origin or a secondary translocation from an already invaded country (e.g. Italy or Germany, two of the largest trading partners of Greece). Hosting a record number of 923 non-native insects (Inghilesi et al. 2013), Italy has been previously identified as the most common origin of alien species for Greece (Avtzis et al. 2017). These statistics may highlight the need for stronger customs control on products imported, not only from Asia and America, but also within Europe. However, it is crucial to take into consideration the cryptogenic nature of a high number of non-native species. As 24% of the presented taxa (112 species) are of unknown origin, perhaps even native to the country, the presented results regarding the origin of species should be viewed with healthy scepticism.

The enigmatic origin of cryptogenic species has been viewed as an impediment to the study of biological invasions, possibly obscuring the true impacts, dispersal corridors, success rate, susceptibility or resistance of communities to invasions and management of non-native taxa (Carlton 1996; Jarić et al. 2019). Our results show that almost a quarter of the catalogued species are cryptogenic (Table 3). Information on the true origin of these species might have altered the overall result, such as the main geographical origin of alien insects in the country.

### Intentional or unintentional introduction

Deliberate introductions of insects in Greece reflect on releases for the biological control of crop pests. More than half of intentionally introduced species are Hymenoptera, mostly parasitoid wasps of the Chalcidoidea superfamily. Introduced Hymenoptera have long been used in Greece for the biological control of aphids (Aphididae), whiteflies (Aleyrodidae) and scales (Coccoidea) (Paraskakis et al. 1980; Kavallieratos et al. 2001; Japoshvili et al. 2010), pests which are responsible for economic losses in agriculture (i.e. through reduced performance of plants, reduced harvest and succession of secondary fungal or viral infections; Perdikis et al. 2008). Furthermore, aphids and scales have been the target of biological control using lady bugs (Coccinellidae), including, but not restricted to, the Australian ladybugs *Cryptolaemus montrouzieri* Mulsant, 1850 and *Rodolia cardinalis* (Mulsant, 1850) (Roy and Migeon 2010). This family contains all six, deliberately introduced alien beetle species in Greece.

Considering Hemiptera, *Perillus bioculatus* (Fabricius, 1775) has been the only deliberately imported, alien true bug species in the country. During the 1950s and 60s, the species was imported to various European countries (including Greece) for the biological control of the Colorado potato beetle *Leptinotarsa decemlineata* Say, 1824 (Mentzelos 1967; Jermy 1980). Although the species failed to establish at the time, since the beginning of the 21<sup>st</sup> century, it started re-appearing in Bulgaria, Greece, Moldova and Serbia (Péricart 2010; Protić and Živić 2012; Valeriu and Elisovețcaia 2014). Whether the presence of *P. bioculatus* in Europe is a result of subsequent accidental introduction events or a previously unnoticed, successful introduction is yet to be clarified.

# Regime

Within the broad feeding guilds, certain orders seem to prevail. Specifically, Hemiptera constitute more than 50% of phytophagous insects. This may highlight the infliction of serious damage to the native Greek flora, as recorded for the majority of economically important taxa. However, regarding native plant species, these impacts have been largely unnoticed. For example, as the oak lace bug *Corythucha arcuata* (Say, 1832) was expanding rapidly in the last 20 years all over Europe (Csóka et al. 2020), the long-term impact of this expansion remained unknown and even partly underestimated. However, recent studies have shown that chronic infestations can have a negative impact on the growth and fecundity of oak stands (Franjević et al. 2018; Paulin et al. 2020). Coupled with the general oak decline syndrome that is observed in Europe (Gallego et al. 1999; Mitchell et al. 2019), the impact of *C. arcuata* on oak stands is expected to become even more severe.

Moreover, Hymenoptera seem to dominate parasitic-predacious insects, while Coleoptera contribute to the majority of detritivorous species (Table 4). Considering that the largest percentage of alien terrestrial arthropods are introduced to Europe through the horticultural/ornamental pathway (Rabitsch 2010), the presence of primarily phytophagous non-native insects in the country underlines the necessity of stricter phytosanitary measures in border and customs control, as well as compliance with the International Standards for Phytosanitary Measures (ISPMs), adopted by the Commission on Phytosanitary Measures (CPM).

# Conclusions

Though the increase in the number of species non-native to Greece by approximately 80% since their first report (Avtzis et al. 2017) can well be attributed to both previously omitted and new records, it is apparent that invasive insects are steadily becoming a major threat to tackle, both at national and international level. Greece, in particular, situated at the crossroads between Asia, Europe and Africa, is likely to experience pressure from various origins, as demonstrated in the current study. In anticipation of the rise in emerging alien species, the establishment of an efficient early warning system seems to be the only plausible approach, particularly if this is integrated in the current phytosanitary surveys. Only by doing this, supplemented with a frequent and exhaustive update of the checklist of the non-native entomofauna of Greece, is it likely to mitigate the impact of these species in the years to come.

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### **Author's contribution**

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# Supplementary material I

#### Comprehensive catalogue of the non-native insect fauna of Greece

Authors: Jakovos Demetriou, Konstantinos Kalaentzis, Christos Kazilas, Evangelos Koutsoukos, Dimitrios N. Avtzis, Christos Georgiadis

Data type: species data

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RESEARCH ARTICLE



# Genic introgression from an invasive exotic fungal forest pathogen increases the establishment potential of a sibling native pathogen

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#### Abstract

Significant hybridization between the invasive North American fungal plant pathogen Heterobasidion irregulare and its Eurasian sister species H. annosum is ongoing in Italy. Whole genomes of nine natural hybrids were sequenced, assembled and compared with those of three genotypes each of the two parental species. Genetic relationships among hybrids and their level of admixture were determined. A multi-approach pipeline was used to assign introgressed genomic blocks to each of the two species. Alleles that introgressed from H. irregulare to H. annosum were associated with pathways putatively related to saprobic processes, while alleles that introgressed from the native to the invasive species were mainly linked to gene regulation. There was no overlap of allele categories introgressed in the two directions. Phenotypic experiments documented a fitness increase in H. annosum genotypes characterized by introgression of alleles from the invasive species, supporting the hypothesis that hybridization results in putatively adaptive introgression. Conversely, introgression from the native into the exotic species appeared to be driven by selection on genes favoring genome stability. Since the introgression of specific alleles from the exotic H. irregulare into the native H. annosum increased the invasiveness of the latter species, we propose that two invasions may be co-occurring: the first one by genotypes of the exotic species, and the second one by alleles belonging to the exotic species. Given that H. irregulare represents a threat to European forests, monitoring programs need to track not only exotic genotypes in native forest stands, but also exotic alleles introgressed in native genotypes.

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#### **Keywords**

Biological invasions, fungi, Heterobasidion, hybridization

#### Introduction

Introgression, i.e. the exchange of genetic material between interfertile species through hybridization, is recognized as a significant catalyst of evolution of eukaryotes (Arnold et al. 2008; Edelman et al. 2019). Thanks to increasing world trade, to advances in genetic characterization and to the democratization of genomic research technologies, the frequency and estimates of hybridization events across all phyla have risen exponentially in recent years (Rieseberg et al. 2000; Barton 2001; Seehausen 2004; Mallet 2005; Le Gac and Giraud 2008; Dasmahapatra et al. 2012; Clarkson et al. 2014).

Although the interspecific transfer of genetic material can be a stochastic process not necessarily involving genes responsible for phenotypic adaptive variation (Suarez-Gonzalez et al. 2018), there are many cases in which introgressive events have been documented to provide novel genes and alleles or to generate favorable allelic combinations that confer adaptive advantages to the recipient organism (Suarez-Gonzalez et al. 2018). Introgression may also increase the fitness of hybrids, allowing them to be competitive against parental species, particularly in novel ecological niches (Mallet 2007), thus facilitating successful invasions (Schierenbeck and Ellstrand 2009; Lee 2002). In plants, it has been well documented that hybridization may result in evolutionary changes increasing invasiveness (Schierenbeck and Ellstrand 2009; Abbott et al. 2003). It is also recognized that hybridization can lead to the emergence of new pathogenic fungal species (Brasier 2000, 2001; Fisher et al. 2012; Giraud et al. 2008; Gladieux et al. 2014; Stuckenbrock 2016).

Stukenbrock (2016) suggested that studies integrating genomic and experimental data are pivotal to understand the evolution of fungal hybrids in nature. Whereas fungal hybrid genotypes can be generated and studied *in vitro* (Olson and Stenlid 2001; Giordano et al. 2018), hybrids resulting from natural selection are difficult to find in nature, given that only a few cases of ongoing hybridization are known (Garbelotto et al. 1996; Gonthier and Garbelotto 2011; Hughes et al. 2013; Pryszcz et al. 2014; Menardo et al. 2016; Sillo et al. 2019).

The fungus *Heterobasidion irregulare* Garbel. & Otrosina is a pathogen of pines in North America that was accidentally introduced into central Italy in 1944, during World War II (Gonthier et al. 2004). The pathogen has become invasive, (Gonthier et al. 2014), and is now included in the A2 list of organisms recommended for regulation by the European and Mediterranean Plant Protection Organization (EPPO 2015). In Central Italy, where *H. irregulare* has been reported so far, its Eurasian sister species *H. annosum* (Fr.) Bref. is also present; however the invasive exotic species is significantly more abundant than the native one (Gonthier et al. 2007). The invasiveness of the exotic species has been experimentally shown to depend not on pathogenicity, which is similar between the two species (Garbelotto et al. 2010), but on its greater ability to saprobically colonize wood and to produce more fruiting bodies, thus increasing its sporulation rate compared to the native sister species (Giordano et al. 2014). Primary infection by *Heterobasidion* is indeed effected by basidiospores colonizing exposed woody surfaces of stumps and wind-thrown trees (Garbelotto and Gonthier 2013), hence an enhanced ability to saprobically colonize wood would confer significant advantages to individuals carrying them. Genomic analyses have corroborated the above hypothesis by showing that genes involved in saprobic growth are significantly more divergent between the two species than expected by genetic drift, while, on the contrary, genes involved in pathogenicity appear to be rather conserved between the two, having undergone purifying selection (Sillo et al. 2015). A recent phenotypic and transcriptomic characterization of artificial *Heterobasidion* hybrids has also determined that *H. irregulare* nuclear genes related to nucleus-mitochondrion communication may confer an advantage to hybrid genotypes compared to *H. annosum* genes involved in the same function (Giordano et al. 2018).

The two species have evolved allopatrically for 34–41 millions of years, but have remained interfertile (Garbelotto and Gonthier 2013). Their recent sympatry has resulted in one of the few known current examples of fungal hybrid swarms (Gonthier and Garbelotto 2011). A study of over 260 genotypes from the hybrid zone in Italy using AFLP and multi gene phylogenies has indicated that, depending on site, up to 42% of the genotypes had an admixed genome, with levels of genomic admixing varying between 5% and 45% (Gonthier and Garbelotto 2011). Sequencing of 11 loci has indicated that in 85% of cases, introgression involved alleles of one species being absorbed by the other, while intragenic recombination was detected only in 15% of cases (Gonthier and Garbelotto 2011). Thus, it is expected that the introgression of whole alleles, and not recombination, may be the dominant process reshaping genomes through hybridization between *Heterobasidion* species. This study may confirm or disprove such expectations.

Two studies using different genetic markers (Gonthier and Garbelotto 2011; Garbelotto et al. 2013) have shown that there is no significant genetic structuring within the invasive and the native species (Gonthier et al. 2014, 2015). Thus, despite the relatively large area of the hybrid zone, it can be assumed that a single population of *H. irregulare* is hybridizing with a single population of *H. annosum*. The lack of population structure further simplifies assignment of alleles to either species, given that a genome-wide comparative study has determined that interspecific DNA divergence between the two taxa is high and ranges between 20.0 and 40.0 SNPs/Kbp, while intraspecific divergence within either species ranges only between 1.0 and 4.0 SNPs/ Kbp (Sillo et al. 2015).

The model system represented by these interbreeding species with a comparable biology and epidemiology provides a unique opportunity to study the genomics of hybridization and introgression in a natural habitat. The main overarching goal of this study was to investigate a fungal invasion at the gene, rather than at the species level (Petit 2004). Theory predicts that the number of introgression events should be significantly larger from the native into the exotic species, if the latter is in demographic expansion (Currat et al. 2008; Excoffier et al. 2009). Despite its relatively recent introduction, the exotic fungus is overwhelmingly dominant and its geographic range is increasing, hence, it can be safely assumed that it is in demographic expansion. Our first prediction, thus, is that the number of chromosomal blocks introgressed from the native into the exotic species should be larger than the number of chromosomal blocks introgressed from the exotic into the native species.

Theory predicts that the introgression of alleles between species may be driven by the adaptive advantages such alleles may provide (Clarke et al. 2002; Fitzpatrick et al. 2010). In our case, invasiveness of the exotic species has been associated with genes involved in saprobic wood decay and in nucleus-mitochondrion communication, hence our second prediction is that alleles of genes involved in these two functions would be introgressed from the exotic into the native species, and that, vice versa alleles involved in these two functions would not be lost by *H. irregulare* when hybridizing with *H. annosum*.

Although we have yet to identify *H. annosum* alleles that may provide an advantage to *H. irregulare* genotypes acquiring them, we can hypothesize that alleles of regulatory genes evolutionarily adapted to the Italian landscape, or that genes putatively related to hybrid genome stability may be good candidates as alleles introgressing into *H. irregulare*. The latter group of alleles may enhance the survival of these hybrids by counteracting the effects of genomic instability caused by the large number of introgression events, as has been shown in other cases (e.g. in animals; Walsh et al. 2018),

Finally, our last prediction is that *H. annosum* genotypes that have absorbed *H. irregulare* alleles involved in saprobic decay and nucleus-mitochondrial communication should display an increased saprobic ability when grown on woody substrates. Conversely, given that virulence does not differentiate the two species, there should be no clear association between genomes of hybrids and this trait. Nonetheless, because all genotypes tested in this study were established in nature and thus naturally viable, we predict pathogenicity should be within the range displayed by pure parental genotypes. In addition, *H. irregulare* genotypes that have absorbed a large number of chromosomal blocks from *H. annosum* may display decreased saprobic growth, pathogenicity or both, due to the effects of large-scale structural genomic rearrangements caused by the large extent of genetic introgression from *H. annosum*.

As stated above, the main overarching goal of this study was to investigate a fungal invasion at the gene level, rather than at the species level (Petit 2004). Are exotic alleles introgressed in the native species, and do these alleles confer an adaptive advantage to genotypes of the native species that acquire them? Given that the exotic fungal species is a forest pathogen that has been identified as a serious threat to the European region and that a strict monitoring program has been recommended for the timely detection of the enlargement of its range (Gonthier et al. 2014; EPPO 2015), this gene centric approach may also indicate whether the monitoring should be expanded to include the detection of specific exotic *H. irregulare* alleles as they introgress in native *H. annosum* populations both within and outside the current zone of infestation by the exotic fungus.

# Materials and methods

# Biological materials and DNA extraction

An extensive sampling of Heterobasidion genotypes was performed in 2005-2006 in the invasion area of *H. irregulare* in Italy where *H. annosum* was also present. Single Heterobasidion basidiospores were collected on woody spore traps following the sampling method of Gonthier et al. (2007). Subsequently, fungal genotypes were identified through taxon-specific PCR (Gonthier et al. 2007) as belonging to H. irregulare or H. annosum and genotyped through Amplified Fragment Length Polymorphism (AFLP) (Gonthier and Garbelotto 2011). Among all sampled genotypes, nine haploid homokaryon hybrid genotypes showing different level of genetic admixture based on the previous AFLP genotyping (Gonthier and Garbelotto 2011) were selected for the current study (Suppl. material 4: Table S1). Mycelia of fungal genotypes were grown in 2% malt extract liquid medium at 25 °C for ten days before being harvested. For the harvesting, approximately 200 mg of fungal mycelium were collected using a vacuum pump, freeze dried overnight and ground using glass beads (diameter 0.2 mm and 0.4 mm) in a FastPrepTM Cell Disrupter (FP220-Qbiogene). Total DNA extraction was performed using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, California, USA), following manufacturer instructions. DNA quantification was carried out by using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA quality was assessed by using a chip-based microcapillary electrophoresis system (Experion, Bio-Rad Laboratories, Hemel Hempstead, UK).

# Sequencing, mapping, and genotype calling

Paired-End (PE) 100bp DNA libraries were prepared for each genotype and sequenced using an Illumina HiSeq2000 platform at the Vincent J. Coates Genomics Sequencing Laboratory (Berkeley, CA, USA). Low quality reads (Q score < 20) as assessed by using FASTQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) were removed by using Trim-Galore! v. 0.5.0 with default parameters (Krueger F. Trim-Galore!, available at http://www.bioinformatics.babraham.ac.uk/projects/trim\_galore/). Reads of each genotype were aligned to the reference H. irregulare TC 32-1 genome (Olson et al. 2012) available in the JGI database using the BWA-MEM algorithm of bwa aligner v. 0.7.15 (r1140) optimized for 100bp PE reads (Li and Durbin 2009), with default parameters. The output was used to generate mapping files in SAM format, which were converted in BAM format, sorted, and indexed by SAMtools v.1.3 (Li et al. 2009). The SNP calling was performed by using the *mpileup* command of SAMtools v.1.3 and the *call* command of bcftools v. 1.10.2 (default parameters). The VCF output files of each genotype were merged into one VCF file which was filtered by using VCFtools 0.1.12b (Danecek et al. 2011) with "--minQ" set to 25 and "--min-meanDP" set to 5. The SNP panels obtained on the six pure genotypes used in the work by Sillo et al. (2015) were also included in the VCF file, for a total of 15 genotypes. In order to prune SNPs affected by linkage

disequilibrium (LD), i.e., to obtain a set of independent SNPs, the software *plink* v1.07 was used. The VCF file containing the SNP panel was first filtered by removing InDels (by using the vcftools command *–remove-indels*) and then converted on *.plink* format by VCFtools. Independent SNPs (unlinked SNPs) were subsequently selected by setting an LD (r2) threshold of 0.8 for SNP pairs through the plink command "*–indep-pairwise*", setting a windows size of 500 bp and a step size of 50. BAM files were submitted to the EMBL database ENA as a project under the PRJEB36378 accession number.

#### Genetic relationship among genotypes

In order to infer the genetic relationship among genotypes and to determine the level of admixture, a phylogenomic analysis and a PCoA were performed on the distance matrix obtained by the unlinked SNP panel of the above mentioned 15 genotypes, i.e. six pure and nine hybrids. Analysis was carried out by using REALPHY v. 112 (Bertels et al. 2014). Reads of each genotype were aligned to the *H. irregulare* reference genome *via bowtie2* (embedded in REALPHY software), and then were used by REALPHY to infer a bootstrap consensus phylogenetic tree (1000 bootstrap replicates, initial tree BioNJ, model of nucleotides substitution GTR) via PhyML (Guindon et al. 2010). The tree was midpoint rooted. The distance matrix was then used as input for a Principal Coordinate Analysis (PCoA) performed by using R.

Results from phylogenomic and PCoA analyses, combined with the outcomes of reads assignment by the software *sppIDer* (see below), were used to decide the taxonomic status of hybrids, i.e. hybrids showing clustering with *H. irregulare* pure genotypes and showing >50% of their genomes as belonging to *H. irregulare* were considered as "*H. irregulare* hybrids", while hybrids showing clustering with *H. annosum* pure genotypes and showing >50% of their genomes as belonging to *H. annosum* were considered as "*H. annosum* hybrids".

# Detection of introgressed regions and identification of putative introgressed alleles

In order to detect regions putatively introgressed from one to the other parental species in hybrid genotypes, i.e. from *H. irregulare* into *H. annosum* and vice versa, we used a conservative approach informed by the outcomes of three distinct analyses, in order to reduce any bias due to the use of any single test for introgression. Tests were done separately for the two groups of hybrid genotypes, that is the group including the five *H. irregulare* genotypes receiving alleles introgressed from *H. annosum*, and the four *H. annosum* genotypes receiving alleles introgressed from *H. irregulare* (see results).

First, the genome-wide four taxon Patterson's D statistics (ABBA/BABA test) was used to detect hybridization events along genomes. The ABBA/BABA test compares patterns of ancestral and derived alleles within four taxa. Equal numbers of sites for each pattern are expected if gene flow is absent. A positive/negative value of D is generally related to an excess of ABBA/BABA sites and might indicate introgression. In



**Figure 1.** Phylogenomic analysis of the nine hybrid genotypes and PCoA **A** Phylogenomic relationship within reference genome, pure and hybrid genotypes as inferred by REALPHY. Bootstrap values higher than 50 % are showed **B** PCoA on the distance matrix obtained by analysis of unlinked SNPs. Red and blue dots represent pure *H. irregulare* and *H. annosum* genotypes, respectively. Pink and cyan dots represents hybrid genotypes assigned as *H. irregulare* and *H. annosum*, respectively. Clusters were circled as follows: red for *H. irregulare* cluster, blue for *H. annosum*, and gray (dashed line) for F1 hybrids.

detail, the test was performed on each of the 14 scaffolds for each hybrid genotype by *angsd* v.0.700 (http://www.popgen.dk/angsd/) using the following parameters: -doAbbababa 1 -doCounts 1. The parameter *-blocksize* was adjusted according to the dimension in bp of each scaffold. Input files were the BAM files obtained from the alignment of reads against the reference genome. The four-taxon D-statistic test was computed using the following combination: *H. annosum* (P1), hybrid genotypes (P2), *H. irregulare* (P3), and *H. occidentale* (O) as outgroup (*H. occidentale* genotype UC1935443 – Holotype at the Berkeley herbarium - which was sequenced and aligned to the reference *H. irregulare* using the same bioinformatic pipeline performed for the other 15 genotypes). The significance of statistics was assessed using the method of weighted block jackknifing.

Second, reads obtained from the hybrid genotypes were aligned on two reference genomes of the parental species, reconstructed by starting from *de novo* assembly of six pure genotypes published by Sillo et al. (2015). The software *sppIDer* (Langdon et al. 2018) was used to accomplish this task. This software maps the reads of hybrids to a concatenated genome built from the reference genomes of the two parental species, to assess the genomic contribution of *H. irregulare* and *H. annosum* in hybrid genotypes. In order to build the combination reference genome, *de novo* assembly of three pure *H. irregulare* and three *H. annosum* genotypes were used to create two distinct consensus genomes of the two parental species. *De novo* assembly of reads from the three pure genotypes of the two parental species was performed using Velvet v.1.2.08 (Zerbino and Birney 2008) with optimized k-mer = 39 bp, and refined by using IMAGE2 (https://

sourceforge.net/projects/image2/), which was set up at twenty iterations, and used as inputs both unassembled reads and contigs in FASTA format generated by the assembly process (Tsai et al. 2010). The software Contiguator v. 2.7.4 (Galardini et al. 2011) was used to perform the final assembly in order to reconstruct the 14 scaffolds for each genotype. A consensus reference genome for *H. irregulare* and for *H. annosum* was built in order to take into account the intraspecific diversity within species. Indexed VCF files with SNPs of pure genotypes from Sillo et al. (2015) were used as inputs, along with *de novo* refined assemblies, for *bcftools* v. 1.10.2 *consensus* command and a single *consensus* fasta file for each species was obtained. The two consensus genomes were concatenated by using the script *combineRefGenomes.py* embedded in *sppIDer*.

Third, to assess the presence and dimension of introgressed blocks, we used the R package *introgress* v. 1.2.3. (Gompert and Buerkle 2010) on the panel of unlinked SNPs (VCF file) previously obtained. The package was used to generate a graphical representation of SNP ancestry allowing for inspecting the different patterns of introgression. *De novo* assemblies were used to assign the introgressed blocks to one species or the other, in order to quantify both in size and in number the introgression events. Two-tailed T-tests were used to determine the significance of the difference between the average size of introgressed blocks of the two species, respectively, as the Shapiro-Wilk normality test for size of blocks confirmed the normal distribution of this dataset. A Wilcoxon rank sum test (Mann–Whitney U test) was used to assess the significance of difference in the number of introgressed blocks of the two species, as the Shapiro-Wilk normality test for number of blocks confirmed the normal distribution of this dataset. All tests were performed with R language.

In H. annosum hybrid genotypes with different levels of admixture caused by different levels of introgression from *H. irregulare*, regions in scaffolds showing excess of ABBA sites and aligning with H. irregulare genome were considered to be regions putatively introgressed from the invasive species to the native. In H. irregulare hybrid genotypes, regions in scaffolds showing excess of BABA excess and aligning with H. annosum genome were considered to be regions putatively introgressed from the native species to the invasive. Bed files resulting from the alignment of hybrid genotype reads to the concatenated reference genome were used to assign reads to one species or the other, and to extract fasta formatted aligned sequences (through the bedtools v. 2.19 getfasta command; Quinlan and Hall 2010). These sequences were searched through blastn algorithm (v. 2.2.31) in the gene catalogue of the established manual annotated genome of *H. irregulare* TC32-1 (Olson et al. 2012) with e-value < 0.05 and nucleotide identity > 98%, in order to identify putative genes in the regions assigned to the two different parental species. Related transcripts were identified by ID, and the sequences of their corresponding encoded proteins were analyzed with Blast2GO v.4.1.9 (Conesa et al. 2005) to search for homologs and to determine their Gene Ontology (GO). At the same time, all gene models of the *H. irregulare* reference genome were annotated through Blast2GO v.4.1.9. Fisher enrichment test (False Discovery Rate < 0.05) was performed with Blast2GO v.4.1.9 to search significant differences in frequencies of GO terms compared to all *H. irregulare* gene models (over-representation).

#### Phenotypic experiment testing virulence and saprobic ability

In order to assess the fitness of hybrid genotypes relative to the fitness of pure genotypes of each one the two species, virulence on host plant and saprobic growth on wood substrate were assessed by means of two distinct phenotypic experiments. All genotypes collected in the invasion area in Central Italy (nine hybrids and four pure genotypes) were used in the assays. For both assays, genotypes were first cultured in Petri dishes filled with Malt Extract Agar (MEA; malt extract agar 33.6 g/L).

The virulence assay was performed by inoculating *P. pinea* germlings, previously grown in sterile conditions, in a microcosm by using a novel and optimized method. Healthy and uniform seeds of P. pinea removed from the cones were washed for 10 minutes in running water, the surface was disinfected for 60 minutes in 30% hydrogen peroxide solution, rinsed twice (10 minutes each) with sterile distilled water and deprived of their lignified tegument (free seeds). Subsequently, they were aseptically transferred into glass jars containing 100 mL of water-agar medium (15 g agar, 1 L distilled water). One seed was inserted in each glass jar. After fifteen days at room temperature ( $25 \pm 2$  °C), germlings were inoculated by placing on the medium close to their root collar two mycelial plugs (6 mm diameter) obtained from the edges of actively growing fungal cultures. Eight replicates for each fungal genotype were prepared and incubated at room temperature ( $25 \pm 2$  °C). As negative controls, eight additional non-inoculated germlings were grown in the same conditions; they remained alive and uncontaminated until the end of the experiment. The germlings were inspected daily for the presence of disease symptoms, including root browning (visible through the agar), needle discoloration, decline in vitality and collapse. The virulence of fungal genotypes was determined on the base of rapidity to death, expressed as the number of days elapsed since pathogen inoculation.

The saprobic assay was performed by measuring the *in vitro* growth rate of each genotype on agar medium mixed with *P. pinea* sawdust to simulate the natural wood substrate as described by Giordano et al. (2018). Sawdust was prepared by cutting a fresh log using a circular saw and collecting the resulting mixture of heart- and sap-wood sawdust in a plastic bag. Petri dishes (90 mm diameter) filled with *P. pinea* sawdust and water agar (15 g sawdust, 12 g agar, 1 L distilled water) were inoculated in the center with a single mycelial plug (6 mm diameter) obtained from the edges of actively growing fungal cultures. Ten replicates for each fungal genotype were incubated at  $25 \pm 2$  °C in the dark. The radial growth of fungal cultures was measured every 48 hours for 10 days along two perpendicular lines. Growth curves from the saprobic assay were compared by performing the permutation test based on t-statistics embedded in the *statmod* R package. The number of permutations was set at 100.

To compare the performance of fungal genotypes in both assays, a pairwise distance matrix of measurements between all possible pairs of genotypes was calculated. For the virulence assay, the distance between two genotypes was calculated as the difference between the average number of days elapsed since pathogen inoculation and death of the germling. For the saprobic assay, the distance between two genotypes was calculated as the difference between the average radial growth at the end of the experiment. Matrices were used as input for Principal Coordinates Analysis (PCoA) performed by using R v.4.0.3 (function *pcoa*).

# Results

# Sequencing, mapping, and genotype calling

The sequenced reads for each genotype were around 8 million (estimate coverage ranging from 17X to 21X). The proportion of reads that aligned to the reference *H. irregulare* genome ranged from 76.20% to 83.14% in four genotypes (118NB, 144SE, 45EH, 49OE), while in five genotypes (136SE, 41NB, 150EA, 115OG and 51ED) ranged from 89.38% to 91.38% (Suppl. material 4: Table S1).

# Phylogenomic and PCoA analyses

The phylogenomic analysis generated a consensus midpoint rooted tree separating two distinct clades, one including pure genotypes of *H. irregulare* and five hybrids (136SE, 41NB, 115OG, 51ED, and 150EA), and one including pure genotypes of *H. annosum* and four hybrids (144SE, 45EH, 118NB, and 49OE).

In the PCoA, *H.annosum* genotypes were distinguishable from *H. irregulare* genotypes by their position along the PC1. However, an additional third cluster was visible within the *H. annosum* cluster, distinguishable from the other two clusters by its position along the PC2. This third cluster included two F1 hybrid genotypes containing comparable levels of the genomes of both species, but still harboring a majority of *H. annosum* SNPs. Genotype 150EA, a putative F1 hybrid harboring a majority of *H. irregulare* SNPs, was in an intermediate position, belonging to both the *H. irregulare* cluster and the cluster including the other two F1 hybrid genotypes.

# Detection of introgressed regions and identification of putative introgressed alleles

In order to detect which alleles were consistently transferred between species and to compare introgression levels in the two directions, we focused on the identification of those alleles that were present in all hybrid genotypes belonging to each one of the two groups of hybrids, by intersecting the outcomes of three distinct approaches.

The ABBA/BABA test performed on each scaffold identified an excess of ABBA and BABA sites that can be interpreted as a footprint of introgressive events. On average, an excess of ABBA sites was detected. ABBA/BABA test results across genomes are shown in Figure 2. The excess of ABBA sites was detected in almost all scaffolds, with the exception of scaffolds 2, 7, 12 and 14, suggesting a genome-wide mosaic architecture of hybrid genomes. Interestingly, two blocks important for pathogenic



**Figure 2.** Results of Patterson D statistics (ABBA/BABA test) along scaffolds for all hybrid genotypes. For the four taxon test, population were as follows: *H. annosum* (P1), hybrid genotypes (P2), *H. irregulare* (P3), and *H. occidentale* (O) as an outgroup. ABBA excess D>0 indicate the level of introgression between *H. irregulare* and hybrid genotypes; BABA excess D<0 indicate the level of introgression between *H. annosum* and hybrid genotypes. Gray line represent average D along the 14 scaffolds.

interactions with Norway spruce and Scots pine have been identified in scaffold 12 (Olson et al. 2012).

The *sppIDer* analysis correctly assigned genomic regions of hybrids to one of the two parental species and is visualized in Suppl. material 3: Figure S1. Detailed alignments are reported in Suppl. material 3: Figures S2–10 (a representative example is reported in Figure 3). Thus, this analysis not only identifies the species of origin of all genomic regions in admixed genomes but also confirmed the species assignment of hybrids, based on the percentage of the inherited parental genome (Suppl. material 3: Figure S1). The package *introgress* was used on a panel of 50 559 unlinked SNPs and identified the number and size of introgressed blocks in both directions, i.e. from the native into the exotic pathogen and from the exotic to the native pathogen. (Figure 4). The number of introgressed blocks per genotype and scaffold is reported in Table 1. Introgressed *H. annosum* blocks in *H. irregulare* hybrid genotypes had a maximum size of 7 007 bp and a minimum size of 21 bp (average size in all 5 *H. irregulare* hybrids = 85.04 bp). The total number of introgressed blocks was 14 088. Conversely, *H. irregulare* blocks into *H. annosum* hybrid genotypes had a maximum



**Figure 3.** Representative example (hybrid genotype 118NB) of whole genome comparative analysis through *sppIDer*. Average depth coverage of reads after the alignment with the two parental species along the 14 scaffolds of the two genomes.

size of 16 536 bp and a minimum size of 64 bp (average size 890.51 bp), and the total number of introgressed blocks was 5 791. In both cases, some scaffolds showed a low number of introgressed blocks, i.e. 6, 10, 12 and 14 (Table 1). When comparing the size of introgressed *H. annosum* blocks *vs.* the size of introgressed *H. irregulare* blocks, the two were significantly different (two-tailed Student T test p-value 2.2 $e^{-16}$ , t = -63.021, df = 19878). The number of introgressed blocks from one species into the other was also different (Wilcoxon rank-sum test p-value = 0.031746, df = 125, Test statistic W = 19). In summary, *H. annosum* hybrids harbored fewer but larger introgressed blocks than *H. irregulare* hybrids.

In *H. irregulare* and *H. annosum* hybrids, the portions of the genome unaffected by introgression were 2 187 985 bp and 7 255 121 bp in size, respectively. These results suggest that intralocus recombination may be occurring only in a minority of the genome, e.g. within a maximum of 2 Mbp and 7 Mbp in *H. irregulare* and *H. annosum*, respectively. Considering all introgression events in all four *H. annosum* hybrid genotypes, a total of 2 384 introgressed *H. irregulare* alleles were found (Suppl. material 1: Dataset S1). A Fisher enrichment test identified GO terms that were overrepresented in this set, including catabolic pathways, i.e. peroxidases, heterocyclic compound binding, mitochondrial transport and vesicle trafficking (Table 2). Conversely, a total of 1 418 introgressed *H. annosum* alleles were found in *H. irregulare* hybrids (Suppl. material 2: Dataset S2). Fisher enrichment tests on this set determined that overrepresented GO terms were mainly related to nuclear functions including RNA processing, gene expression, nucleotide binding (Table 3). No alleles showing the same GO were ever found to be introgressed in both directions between the two species.



**Figure 4.** Analysis of introgressed blocks from native to invasive species (**A**) and from invasive to native species (**B**) by INTROGRESS. The dataset of used SNPs (markers) are ordered in x-axis based on genomic locations. In (A), Blue rectangles represent marker assigned to *H. annosum*. Plot on the right represents the fraction of the hybrid genome inherited from *H. irregulare* for each genotype defined as hybrid index. Three pure genotypes of *H. annosum* (on the top) are also included in the analysis. In (B), red rectangles represent marker assigned to *H. irregulare*. Plot on the right represents the fraction of the hybrid genome inherited from *H. irregulare*. Plot on the right represents the fraction of the hybrid genome inherited from *H. irregulare*. Plot on the right represents the fraction of the hybrid genome inherited from *H. irregulare* for each genotype defined as hybrid index. Three pure genotypes of *H. irregulare* for each genotype defined as hybrid index. Three pure genotypes of *H. irregulare* for each genotype defined as hybrid index. Three pure genotypes of *H. irregulare* for each genotype defined as hybrid index. Three pure genotypes of *H. irregulare* for each genotype defined as hybrid index. Three pure genotypes of *H. irregulare* (on the top) are also included.

		Numbe	r of intro	oressed	blocks fro	m	N	umber of	introgres	sed bloc	ks from H	. annosus	<i>m</i> into
		H. irregulare into H. annosum hybrids					H. irregulare hybrids						
Scaffold	ID	118NB	490E	45EH	144SE	Sum	ID	136SE	41NB	51ED	1150G	150EA	Sum
scaffold_01		118	50	36	491	695		139	121	90	130	186	666
scaffold_02		254	51	48	202	555		165	249	315	233	274	1236
scaffold_03		243	39	44	47	373		154	281	1171	237	225	2068
scaffold_04		476	33	42	165	716		302	492	161	438	164	1557
scaffold_05		159	35	26	43	263		190	378	73	327	220	1188
scaffold_06		67	25	30	119	241		44	77	320	87	49	577
scaffold_07		204	29	41	72	346		124	226	448	220	53	1071
scaffold_08		209	28	31	189	457		108	152	145	179	272	856
scaffold_09		238	24	26	129	417		118	228	69	243	100	758
scaffold_10		461	31	29	65	586		111	192	188	176	101	768
scaffold_11		178	26	24	136	364		113	206	220	242	109	890
scaffold_12		214	27	26	16	283		82	143	330	157	50	762
scaffold_13		119	23	18	92	252		103	163	499	115	177	1057
scaffold_14		179	22	16	26	243		104	111	91	167	161	634

Table 1. Summary of number of introgressed blocks in *Heterobasidion* hybrids.

GO-ID	Term	Category	FDR	Sequences (N°) in Test Group	Sequences (N°) in Reference Group
GO:0016209	antioxidant activity	MOLECULAR_FUNCTION	3.405E-03	240	586
GO:0003824	catalytic activity	MOLECULAR_FUNCTION	6.360E-13	996	2531
GO:0043169	cation binding	MOLECULAR_FUNCTION	3.305E-02	147	351
GO:0030130	clathrin coat of trans-Golgi network vesicle	CELLULAR_COMPONENT	2.151E-02	136	314
GO:0051234	establishment of localization	BIOLOGICAL_PROCESS	1.651E-04	214	479
GO:1901363	heterocyclic compound binding	MOLECULAR_FUNCTION	1.850E-08	700	1781
GO:0016787	hydrolase activity	MOLECULAR_FUNCTION	1.918E-04	363	904
GO:0051536	iron-sulfur cluster binding	MOLECULAR_FUNCTION	2.151E-02	20	21
GO:0016020	membrane	CELLULAR_COMPONENT	6.407E-05	263	605
GO:0051540	metal cluster binding	MOLECULAR_FUNCTION	2.151E-02	20	21
GO:0046872	metal ion binding	MOLECULAR_FUNCTION	2.993E-02	275	722
GO:0006850	mitochondrial pyruvate trans- port	BIOLOGICAL_PROCESS	6.854E-03	328	853
GO:0006839	mitochondrial transport	BIOLOGICAL_PROCESS	3.365E-03	179	413
GO:0006807	nitrogen compound metabolic process	BIOLOGICAL_PROCESS	1.976E-03	548	1494
GO:0097159	organic cyclic compound binding	MOLECULAR_FUNCTION	1.850E-08	700	1781
GO:0097159	organic cyclic compound binding	MOLECULAR_FUNCTION	4.798E-02	79	168
GO:0071704	organic substance metabolic process	BIOLOGICAL_PROCESS	6.213E-07	695	1813
GO:0055114	oxidation-reduction process	BIOLOGICAL_PROCESS	3.011E-03	240	584
GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	MOLECULAR_FUNCTION	3.249E-02	112	253
GO:0004601	peroxidase activity	MOLECULAR_FUNCTION	2.404E-02	57	105
GO:0016462	pyrophosphatase activity	MOLECULAR_FUNCTION	1.943E-03	106	211
GO:0030140	trans-Golgi network transport vesicle	CELLULAR_COMPONENT	4.263E-03	178	413
GO:0012510	trans-Golgi network transport vesicle membrane	CELLULAR_COMPONENT	6.854E-03	139	310
GO:0055085	transmembrane transport	BIOLOGICAL_PROCESS	2.009E-03	123	256
GO:0006810	transport	BIOLOGICAL_PROCESS	1.421E-04	214	477

**Table 2.** Gene Ontology terms over-represented in the dataset of 2,384 introgressed *H. irregulare* alleles in hybrid *H. annosum* genotypes. FDR: False Discovery Rate (FDR) corrected p-value.

# Virulence and saprobic ability of fungal genotypes

In the virulence assay, *in vitro* mortality of pine germlings was recorded for a period of 40 days. Forty days post inoculation (DPI), each fungal genotype had killed all germlings it had been inoculated onto, but there was significant variability among genotypes in the average DPI needed to kill all germlings. Seven virulent genotypes (one pure *H. annosum* and six hybrids) were able to induce damping-off of germlings within 20 DPI (Suppl. material 4: Table S2). By contrast, three genotypes (one pure *H. irregulare* and two hybrids) killed germlings after 30 DPI. PCoA on

GO-ID	Term	Category	FDR	Sequences (N°)	Sequences (N°) in
				in Test Group	Reference Group
GO:0008152	metabolic process	BIOLOGICAL_PROCESS	4.201E-10	824	3403
GO:0000166	nucleotide binding	MOLECULAR_FUNCTION	5.605E-07	258	886
GO:0032553	ribonucleotide binding	MOLECULAR_FUNCTION	1.131E-04	193	670
GO:0009987	cellular process	BIOLOGICAL_PROCESS	1.166E-04	576	2457
GO:0005488	Binding	MOLECULAR_FUNCTION	1.342E-04	641	2781
GO:0032555	purine ribonucleotide binding	MOLECULAR_FUNCTION	2.269E-04	184	646
GO:0035639	purine ribonucleoside triphos- phate binding	MOLECULAR_FUNCTION	2.269E-04	183	641
GO:0017076	purine nucleotide binding	MOLECULAR_FUNCTION	2.961E-04	185	655
GO:0043167	ion binding	MOLECULAR_FUNCTION	4.752E-04	461	1942
GO:0005622	intracellular	CELLULAR_COMPONENT	7.746E-04	266	1033
GO:0005524	ATP binding	MOLECULAR_FUNCTION	8.246E-04	158	552
GO:0032559	adenyl ribonucleotide binding	MOLECULAR_FUNCTION	8.330E-04	159	557
GO:0010467	gene expression	BIOLOGICAL_PROCESS	9.074E-04	151	524
GO:0030554	adenyl nucleotide binding	MOLECULAR_FUNCTION	1.122E-03	160	566
GO:0043039	tRNA aminoacylation	BIOLOGICAL_PROCESS	1.529E-03	17	19
GO:0043038	amino acid activation	BIOLOGICAL_PROCESS	1.529E-03	17	19
GO:0016070	RNA metabolic process	BIOLOGICAL_PROCESS	2.027E-03	122	411
GO:0004812	aminoacyl-tRNA ligase activity	MOLECULAR_FUNCTION	2.537E-03	16	18
GO:0006418	tRNA aminoacylation for protein translation	BIOLOGICAL_PROCESS	2.537E-03	16	18
GO:0019787	ubiquitin-like protein transferase activity	MOLECULAR_FUNCTION	6.277E-03	10	7
GO:0017111	nucleoside-triphosphatase activity	MOLECULAR_FUNCTION	9.330E-03	75	235
GO:0034660	ncRNA metabolic process	BIOLOGICAL_PROCESS	2.207E-02	31	73
GO:0005634	nucleus	CELLULAR_COMPONENT	2.251E-02	122	447
GO:0006399	tRNA metabolic process	BIOLOGICAL_PROCESS	4.406E-02	24	53

**Table 3.** Gene Ontology terms over-represented in the dataset of 1,418 introgressed *H. annosum* alleles in hybrid *H. irregulare* genotypes. FDR: False Discovery Rate (FDR) corrected p-value.

virulence data differentiated two large groups, one including more virulent genotypes and one including less virulent genotypes (Suppl. material 3: Figure S11), however there was no clear correlation between group assignment and genomic background of the genotypes.

In the saprobic assay, the extent of *in vitro* mycelial growth on a wood-rich substrate was assessed for a period of ten days. Only one pure *H. irregulare* genotype (9OA) was able to fully colonize the Petri dishes within the time frame of the experiment, while other genotypes varied in the extent of their growth (Suppl. material 4: Table S3). PCoA on saprobic assay data differentiated genotypes based on their growth rate. Three *H. annosum* hybrids had a growth rate comparable to that of all pure *H. irregulare* genotypes. All the remaining hybrids and the *H. annosum* genotype were placed in a cluster of genotypes characterized by lower growth rates (Figure 5). Permutation tests determined that growth rates of *H. annosum* hybrids were significantly



**Figure 5.** Results of PCoA on phenotypic data obtained from the saprobic assay. Saprobic ability increase from left to right. Red and blue dots represent pure *H. irregulare* and *H. annosum* genotypes, respectively. Pink and cyan dots represents hybrid genotypes assigned as *H. irregulare* and *H. annosum*, respectively.

higher than those of *H. irregulare* hybrids (observed value of the t-statistic = 6.506, p-value adjusted for multiple testing = 0.00043). The saprobic growth of *H. annosum* hybrids was no different from that of *H. irregulare* pure genotypes (p-value adjusted for multiple testing > 0.05). On the other hand, the growth rate of *H. irregulare* hybrids was significantly lower than that of pure *H. irregulare* genotypes (observed value of the t-statistic = -2.439, p-value adjusted for multiple testing = 0.000051).

#### Discussion

The introgression of both exotic genotypes and exotic alleles in native populations, where exotic is defined here as pertaining to an exotic species, is knowingly associated with biological invasions. During interspecific hybridization, the interspecific introgression of alleles bears significant evolutionary implications, shifting the focus from the more traditional "species-centric" to a "gene-centric" concept of invasion (Crispo et al. 2011). In this study, we have shed light on the dynamics of hybridization-mediated allelic introgression between an invasive and a native fungal species. We performed

the study by sampling pure and admixed genotypes in an area in Italy where the North American forest pathogen *H. irregulare* has been introduced and is hybridizing with the native Eurasian forest pathogen *H. annosum* (Gonthier et al. 2007; Gonthier and Garbelotto 2011). This system is particularly interesting as the precise date and location of introduction are known (Gonthier et al. 2004), the biology and population structure of pathogens are also known, the two species are fully interfertile, and 15 years of research have identified some of the likely mechanisms behind the clear demographic dominance of the exotic species compared to the native one.

For this study, we selected a set of 15 natural genotypes with levels of admixture ranging between zero (pure genotypes) and 45% (fully admixed) based on a large number of anonymous AFLP markers. The fact that all studied genotypes had successfully established themselves on wood in a natural setting suggested they were all sufficiently fit to survive in nature. Our expectation that these genotypes would display variably admixed genomic regions (Elgvin et al. 2017) and variable phenotypic fitness proved correct, allowing us to associate genomic variability and phenotypic fitness with specific introgression histories.

Phylogenomic analyses confirmed that the selected natural hybrids varied in the level of genomic admixture. Three hybrid genotypes (118NB, 150EA and 49OE) had a balanced level of admixture, suggesting either a recent hybridization event between two pure parental genotypes or balanced backcrosses with both parental species. The remaining six genotypes had signs of introgression in a relatively small portion of their genome, indicative of multiple backcrosses with a single parental species, to which they could be assigned. Some scaffolds (e.g. scaffolds 6, 12 and 14) showed significantly lower levels of introgression, suggesting these scaffolds contain genes regulating essential functions or species-specific genes (Olson et al. 2012).

The genomes of *H. irregulare* hybrids were profoundly affected by introgression from *H. annosum*, and only approximately 2 Mbp appeared to have been left untouched, while the remaining 30 Mbp were subject to allelic replacement. A significantly larger amount (about 7 Mbp) of the genome of *H. annosum* hybrids, instead, had been left untouched by the introgression of *H. irregulare* alleles, suggesting a smaller scale process in terms of genomic rearrangement. Regions not affected by alleles introgression might be subjected to intra locus recombination, however, in this study, these regions were estimated to be only approximately 24% and 7% of the genome in *H. annosum* and *H. irregulare*, respectively. The effects of intralocus recombination, despite their significance, are not treated in this work and these regions were excluded from the quantification of allelic introgression.

The size of blocks introgressed from the exotic *H. irregulare* into the native *H. annosum* was on average one order of magnitude larger than the size of those introgressed from *H. annosum* into *H. irregulare*. Small block dimensions (about 85 bp on average) and high number of genomic blocks (more than 14 000) introgressed into the exotic species may result in the excessive fragmentation of chromosomal blocks. As a result, exons may be partial, non-functional and may be more likely to be lost during recurrent recombination events (Sachdeva and Barton 2018). On the other hand, a low

number (about 6 000) of larger blocks (on average 890 bp in size) introgressed from *H. irregulare* may suggest that putatively adaptive and functional alleles may be truly integrated and preserved into the recipient *H. annosum* genomes. It is known that large genomic blocks have a better chance to be maintained through multiple generations when compared to small blocks (Baird et al. 2003; Sachdeva and Barton 2018).

Alleles contained in the larger blocks introgressed from H. irregulare into H. annosum showed a GO term enrichment related to catabolic pathways, i.e. peroxidases, heme binding proteins, oxidoreductases, metal ion binding proteins and to vesicle trafficking. Oxidoreductases and heme peroxidase genes have been identified among those that best differentiate the highly saprobic *H. irregulare* from the less saprobic *H.* annosum (Sillo et al. 2015), and some of them, e.g. manganese peroxidases, are known to play a role in lignin degradation during saprobic colonization of wood by Heterobasidion species (Yakovlev et al. 2013). All experimental evidence has indicated that higher saprobic potential explains much of the invasiveness of *H. irregulare* (Giordano et al. 2014; Giordano et al. 2019). Another category of over-represented GO terms among H. irregulare alleles introgressed into H. annosum was related to mitochondrial transport. Interestingly, this is another category of genes that well differentiates the two species at the genomic level (Sillo et al. 2015), with *H. irregulare* nuclear genes better at regulating mitochondrial functions in admixed genomes than H. annosum nuclear genes (Sillo et al. 2015). Based on the preexisting literature, the introgression of alleles belonging to these gene groups above into *H. annosum* had been predicted. Alleles involved in clathrin-mediated vesicular trafficking were also significantly enriched among alleles introgressed in H. annosum hybrids: this catabolic process plays a role in the breakdown of host defenses and in the development of polarized fungal hyphal growth (Steinberg 2007; Shultzhaus et al. 2017). To explain the advantages provided by the presence of these alleles, it should be noted that mutants of the fungus Candida albicans (C.P. Robin) Berkhout with modifications in genes affecting vesicular trafficking are plagued by defects in the cytoskeleton (Epp et al. 2010). Finally, alleles related to "iron-sulphur cluster binding" and "membrane" were found to be introgressed in H. annosum. Iron-sulphur cluster enzymes are known to play different roles in cells, including a role in regulating genomic integrity (Stehling et al. 2012) and in detoxification (Haas et al. 2008). Maintaining genomic integrity is obviously an important function in admixed hybrid genomes, while detoxification may be relevant, for example, during delignification processes (Sista Kameshwar and Qin 2020). Moreover, genes related to membrane may affect the activity of extracellular enzymatic activities, required by wood decay fungi to delignify the substrate in which they live (Wu et al. 2020).

Conversely, directional allelic introgression from *H. annosum* to *H. irregulare*, although larger in scale, was limited to DNA-methylation and transcription factor genes. Genes related to DNA-repair have been reported in hybridization events in animals, e.g. sparrows, and it has been hypothesized that they may be selected by nature to modulate epistatic interactions following genomic alterations caused by hybridization (Walsh et al. 2018). The re-patterning of DNA methylation has been also recently

documented in plants (Li et al. 2019). This phenomenon is a process affecting gene expression and silencing of mobile elements in the genome (Martienssen and Colot 2001). The introgression of these alleles can be regarded as the result of a selection of *H. annosum* alleles involved in epistatic regulation within the highly mosaic-like H. irregulare hybrid genotypes. In other words, given that the consequence of mosaic architecture of hybrid genomes can be the alteration of transcriptomic profiling and/ or proliferation of mobile elements, methylation and regulation of genes may contribute to the stability of the hybrid genome. Regulatory genes may also be important in optimizing gene expression and adaptation in a microbial species facing a new environment (Elena and Lenski 2003), and it has been hypothesized that evolution may promote adaptations through already available and even acquired genetic tools rather than generating novel catabolic components (Lavoie et al. 2010). In addition, H. annosum GO terms involved in tRNA aminoacylation processes were identified as being overrepresented in *H. irregulare* hybrids. It is worth noting that tRNA aminoacylation is not only involved in protein translational fidelity that is indispensable for cell survival, but also allows for the tolerance of low levels of mistranslation, a predicament that may occur during stressful exposure to new environments (Wiltrout et al. 2012; Pan 2013). Thus, it could be inferred that the introgression of these alleles may be beneficial to *H. irregulare* hybrids constantly facing exposure to novel environments. A further transcriptomic analysis (i.e. RNA-sequencing) will be pivotal to clarify the functional role of introgressed alleles during different life phases of the hybrids (e.g. wood colonization, infection).

The sabrobic in vitro assay revealed that H. annosum hybrids containing H. irregulare alleles involved in saprobic decay had an increased saprobic ability, comparable to that of pure *H. irregulare* genotypes. Based on the results of the saprobic assay, it is reasonable to hypothesize these H. annosum hybrid genotypes may be competitive against pure H. irregulare genotypes. By contrast, H. irregulare hybrid genotypes showed a decrease in saprobic growth when compared to pure H. irregulare genotypes. We believe the impact of large-scale introgression is costly to hybrid H. irregulare genotypes, resulting in a reduction in fitness. We suggest that viability may be maintained only in those H. irregulare hybrids that: first, absorb transcription and methylation related genes from the native species stabilizing their genomic instability caused by large genomic alterations, and, second, maintain their alleles involved in production of peroxidase and heme-binding proteins, mitochondrial transport and vesicle trafficking. This last point is corroborated by the fact that the alleles involved in catabolic pathways were not affected by introgressive events, i.e. these alleles were never replaced by H. annosum alleles in any of the H. irregulare hybrids. Negative epistatic interactions and the breakdown of advantageous gene complexes (Lynch 1991; Guerrero et al. 2017) in H. irregulare recipient genomes, highly modified by large scale introgression events, are two plausible explanations for this detrimental effect on fitness.

Virulence assays in the past have not been able to differentiate the two species (Garbelotto et al. 2010; Pepori et al. 2019). The results of the virulence assay performed in this study further confirmed that virulence may be a genotype-specific trait, rather than a species-level trait as previously suggested (Garbelotto et al. 2010). We note, though, that all hybrids fell within the range of virulence identified for the pure parental genotypes used in the study, and so all can be regarded as ecologically viable. This result confirms that by using genotypes that have established themselves on wood in a natural setting, we obtained a representative sample of functionally and ecologically viable genotypes. In addition, the result also confirms that hybrid swarms represent a viable genetic bridge for the transfer of genetic material between the two species (Brasier 2001).

Although we recognize that the number of genotypes characterized as pure *H. annosum* and *H. irregulare* used in our phenotypic tests is limited, the genotypes employed here represented a select sample representative of each species, and results were in agreement with our expectations. In a previous comparative genomic study, genes involved in virulence have been reported to be conserved between the two species (Sillo et al. 2015), resulting in indistinguishable virulent phenotypes (Garbelotto et al. 2010; Pepori et al. 2019). Conversely, genes involved in catabolic processes, including wood decay, and genes regulating nucleus-mitochondrion communication have been shown to diverge (Sillo et al. 2015) and to be associated with different phenotypes (Giordano et al. 2014; 2018). Results of this study are in agreement with a role of selection driving introgression: alleles from divergent loci such as those involved in catabolic processes were significantly enriched as a result of introgression and caused a measurable phenotypic change in the species receiving them by increasing its saprobic ability. On the other hand, we could not document introgression-related enrichment of genes directly involved in virulence of live plants and no measurable change in the associated phenotype could be measured.

It is known that competition with parental species living in the same environment may negatively affect the success of hybrids (Stukenbrock 2016). In the pathosystem studied here, the exotic Heterobasidion species may outcompete or even replace the native species, possibly due to its higher saprobic and sporulation potential (Gonthier et al. 2014). Our results suggest that H. annosum genotypes hybridizing with H. irregulare may become as competitive as those belonging to the exotic species, by receiving alleles that provide them with adaptive phenotypic advantages, including an increased saprobic wood decay potential. If this suggested scenario was correct, these alleles introgressed from H. irregulare into H. annosum, including those related to the saprobic ability among others, may move into H. annosum populations both inside and outside the hybrid zone. The rapid absorption of advantageous or adaptive exotic alleles through introgressive hybridization has been previously documented for many organisms (Fitzpatrick et al. 2010; Crispo et al. 2011) including the fungi (Hessenauer et al. 2020). Thus, H. annosum hybrids, with some specific H. irregulare alleles introgressed in their genome, may themselves represent a threat to the stability of European forests. On the other hand, introgression from the native into the invasive species affected a larger portion of the genome and involved alleles exclusively associated with gene regulation and transcription processes, resulting in lower saprobic growth, but ensuring the viability of hybrid genotypes.

# Conclusion

Overall, our results show that mating events leading to viable and fertile *Heterobasidion* hybrids occur frequently in the Latium region of Italy where the exotic forest pathogen *H. irregulare* is now sympatric with the native forest pathogen *H.annosum*. The resulting allelic introgression has identified specific classes of adaptive *H. irregulare* alleles that confer an advantage to recipient native *H. annosum* genotypes. Given the phenotypic outcomes of these introgression events including a measurable increase in saprobic potential of *H. annosum* individuals, we propose an operational shift in the monitoring of the invasion process to include not only the detection of exotic *H. irregulare* alleles or genes spreading into native *H. annosum* populations. A gene centric approach to the study of invasions has been previously advocated (Petit, 2004) and clear adaptive implications of genic introgression between tree pathogens have been previously documented for the two fungi causing Dutch elm disease (Hessenauer et al. 2020).

In conclusion, despite the limited number of sequenced hybrid genotypes, this study suggests that horizontal allelic movement occurred as a result of interspecific hybridization, but it was qualitatively and quantitatively different when comparing the two directions of introgression. A large population genomics survey in and near the invasion area in Central Italy targeting introgressed alleles identified in this study will be pivotal to further validate these results.

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Sequence data were submitted to the EMBL database ENA - European Nucleotide Archive as a project under the PRJEB36378 accession number. All other data were available within the article or its supplementary materials.

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# Supplementary material I

## Dataset S1. List of introgressed H. irregulare alleles

Authors: Fabiano Sillo, Matteo Garbelotto, Luana Giordano, Paolo Gonthier Data type: dataset

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# Supplementary material 2

# Dataset S2. List of introgressed H. annosum alleles

Authors: Fabiano Sillo, Matteo Garbelotto, Luana Giordano, Paolo Gonthier Data type: dataset

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# Supplementary material 3

#### Figures S1–S11

Authors: Fabiano Sillo, Matteo Garbelotto, Luana Giordano, Paolo Gonthier

Data type: images

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# Supplementary material 4

# Tables S1–S4

Authors: Fabiano Sillo, Matteo Garbelotto, Luana Giordano, Paolo Gonthier Data type: tables

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**REVIEW ARTICLE** 



# Diversity and impacts of key grassland and forage arthropod pests in China and New Zealand: An overview of IPM and biosecurity opportunities

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#### Abstract

For both New Zealand and China, agriculture is integral to the economy, supporting primary production in both intensive and extensive farming systems. Grasslands have important ecosystem and biodiversity functions, as well providing valuable grazing for livestock. However, production and persistence of grassland and forage species (e.g. alfalfa) is not only compromised by overgrazing, climate change and habitat fragmentation, but from a range of pests and diseases, which impose considerable costs on growers in lost production and income. Some of these pest species are native, but increasingly, international trade is seeing the rapid spread of exotic and invasive species. New Zealand and China are major trading partners with significant tourist flow between the two countries. This overview examines the importance of grasslands and alfalfa in both countries, the current knowledge on the associated insect pest complex and biocontrol options. Identifying similarities and contrasts in biology and impacts along with some prediction on the impact of invasive insect species, especially under climate change, are possible. However, it is suggested that coordinated longitudinal ecological research, carried out in both countries using sentinel grass and forage species, is critical to addressing gaps in our knowledge of biology and impact of potential pests, along with identifying opportunities for control, particularly using plant resistance or biological control.

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#### **Keywords**

alfalfa, biodiversity, climate change, pathways, invasive alien species, plant biosecurity, sentinel plants, pest risk analysis, international trade

#### Introduction

Grasslands can be defined as "terrestrial ecosystems dominated by herbaceous and shrub vegetation and maintained by fire, grazing, drought and/or freezing temperatures" (White et al. 2000). Natural grassland ecosystems are important for both ecological functioning and biodiversity conservation (Mark et al. 2013; Pfeiffer et al. 2018). They contribute to water supply and management, soil organic C storage, erosion control and climate mitigation (Bengtsson et al. 2019; Sollenberger et al. 2019), as well as supporting extensive faunal communities that often have high levels of endemism (White et al. 2000; Wu et al. 2015). They can also be culturally important, supporting traditional lifestyles and have educational, scientific, recreational and tourism values (Mark et al. 2013). Economically, both natural and modified grasslands are critical for food production (O'Mara 2012).

China (along with Mongolia) hosts some of the world's largest continuous grasslands. These are largely natural swards dominated by Leymus chinensis (Trin.) Tzvel (sheep grass or Chinese lyme grass) with human modification to its composition occurring to a limited extent (Pfeiffer et al. 2018), but with significant habitat degradation due to climate change and overgrazing (Schönbach et al. 2011; Li et al. 2012; Wu et al. 2015; Lv et al. 2019; Zhou et al. 2019). Chinese natural grasslands cover a vast territory (Figure 1) and are conservatively estimated to cover approx. 2.8 M km<sup>2</sup> (Pfeiffer et al. 2018) or ca. 40% of the land area of China. The Provinces of Qinghai, Gansu and the autonomous regions Inner Mongolia (Hulunbuir Prairie and Xilingol Prairie), Xinjiang (Yili grassland) and Tibet (Naqu Alpine grassland) are the main grassland regions in China, with four main ecosystem types: meadow steppe; typical steppe; desert steppe; and alpine steppe (Kang et al. 2007). The Inner Mongolian grassland is classified as temperate steppe, with native species dominated by Cleistogenes squarrosa (Trin.). Ken, L. chinensis and Stipa krylovii Roshev., Artemisia frigida Willd. and Caragana microphylla Lam. (Leguminosea). In addition to grasslands, the introduction of crop species to Inner Mongolia has been used to support livestock, such as horses, sheep, goats and camels (Wu et al. 2015; Pfeiffer et al. 2018).

In New Zealand, grasslands comprise natural areas (variously dominated by native *Poa* spp., *Festuca* spp., *Carex* spp. or *Chionchloa* spp.), modified native grasslands consisting of native and introduced or volunteer exotic species and cultivated and sown pastures consisting almost entirely of exotic grass species (predominantly *Lolium* spp. L.) and legumes (clovers (*Trifolium* L. spp.), *Medicago sativa* L. (alfalfa, lucerne)) (hereafter 'improved grasslands' or pastures). These highly modified environments cover about 0.11 M km<sup>2</sup> and comprise 41% of New Zealand's land area



**Figure 1.** Map of China, showing the geographic distribution of the main grassland types. The four most extensive and important grasslands in China are indicated by (1) temperate meadow-steppe, (2) temperate steppe, (3) temperate dessert-steppe, (4) temperate dessert and (5) alpine meadow.

(Ferguson et al. 2018). The precise composition of improved grasslands depends on climate, farm type and topography and ranges from annual monocultures (e.g. *Festuca perennis* Lam. (formerly *Lolium multiflorum*)) to perennial combinations of generally no more than five sown plant species (e.g. perennial ryegrass (*Lolium perenne* L.), white clover (*Trifolium repens* L.), red clover (*T. pratense* L.), chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.). Perennial legumes form a critical component of improved grasslands, as they fix atmospheric nitrogen (N) and make it available to other plants, reduce the need for artificial N inputs and provide protein for grazing animals (Peoples et al. 2017, 2019).

This overview examines the role that grasslands and the forage crop alfalfa have in both countries, comparing and contrasting land use and management of important insect pests. We focus on species that have been identified from both English and Chinese language literature, as having a significant impact on grassland and forage production systems. Where possible, information on biological control agents, associated with these pests, was also collated. Finally, we discuss approaches to developing a better understanding of potential biosecurity threats which these pest species may have, if trade and tourism pathways facilitate their movement between the two countries and subsequent establishment.

#### Data acquisition

Information on insect pest species was obtained by searching peer-reviewed literature in English and Chinese scientific databases. The databases used were Scopus, CAB Abstracts, Chinese National Knowledge Infrastructure (CNKI) and WanFang Data. Search strategies included English, Latin and Chinese terms. These included generic keywords, such as alfalfa/lucerne and ryegrass, *Lolium spp., Leymus spp.*, New Zealand, China, grassland insect pests, biological control agents and also more specific searches by insect (e.g. *Oedaleus asiaticus, Sminthurus viridis*) or plant (e.g. *L. perenne, L. chinensis*) species. A basic search strategy combined Insect (genus / species) AND Plant (genus / species) AND Country (China / New Zealand). The search results were then analysed and selected for relevancy. If the insect-plant association was not able to be determined, then the item was excluded from the search results. These databases were augmented by internal reports held by the authors' respective research organisations.

#### Grassland and forage ecosystems

#### China

China has a rich diversity of forage plants comprising 2581 species in 364 genera and 65 families, including 57 grass species in 28 genera and 56 legume species in 24 genera. Of these, 14 species are commonly cultivated (Yan et al. 2008). The main commercial forage crops grown in China include *L. chinensis*, of which 1.31 M tonnes are produced per annum (p.a.), *M. sativa* (1.43 M tonnes p.a), *Zea mays* L. (silage corn) (0.53 M tonnes p.a.) and *Lolium* spp. (0.30 M tonnes p.a.) along with *Avena sativa* L. (Oats) (0.47 M tonnes p.a.) (Yun and Song 2017).

In Inner Mongolia and western northeast China, awnless brome (*Bromus inermis* Leyss.) is grown in a range of environments, including forest edge meadows, hill country and riverside roads. The grass has high nutritional value, good palatability, is tolerant to grazing and is cold hardy. It is also used to stabilise erosion-prone sandy areas. Fescue (*Festuca* spp. L.) is grown in alpine meadows, hillside grasslands, forests, thickets and sandy soils at an altitude of 2200-4400 metres above sea level, to provide forage for cattle, sheep and horses. The Xilin River system represents a typical inland river found in Inner Mongolia grassland. Originating in the Keshiketen Banner of Chifeng City, the river flows southeast to northwest, finally feeding into the Chagan Naoer wetland. The Xilin River Basin covers an area of approximately 10,000 km<sup>2</sup> with distinct landscape form and diverse plant communities (Tong et al. 2004). The dominant grass species are *L. chinensis* and *Stipa grandis* P. Smirn, along with *Agropyron cristatum* (L.) Gaertn, *Achnatherum sibiricum* (L.) Keng ex Tzvelev, *Koeleria cristata* (Linn.) Pers., *C. squarrosa* and *Carex korshinskyi* Kom. (Zhang et al. 2014).

In improved dairy and sheep grasslands in China, the main grass species are *Z. mays*, perennial ryegrass L. and annual ryegrass, *Elymus dahuricus* Turcz. ex Griseb and *E. sibiricus* L., *Bromus inermis* Leyss. and cocksfoot (*Dactylis glomerata* L.). The

predominant legumes are *M. sativa*, erect milkvetch (*Astragalus adsurgens* Pall.), common sainfoin (*Onobrychis viciaefolia* Scop.), *T. repens, T. pratense* and common vetch (*Vicia sativa* L.). Oats *Avena sativa* L., sudan grass (*Sorghum sudanense* (Piper) Stapf.) and sorghum (*S. bicolor* (L.) Moench) are also grown for forage. While the economic value of these main forage plant species is not available, the area grown and yields are shown in Suppl. material 1: Table S1.

Alfalfa is perennial leguminous forage, valued in China as a high-quality feed for livestock and poultry. Since 2000, China has been returning farmland to forest and grassland to reduce water and wind erosion in areas prone to these phenomena (Zao et al. 2012). As a result, the area planted in alfalfa has successively expanded as it is regarded as an important part of the restoration of regional ecological environments and because it is seen as integral to the transformation of traditional agricultural, by increasing farmers' income and promoting social and economic development (Zao et al. 2012; Li 2019).

#### New Zealand

Native grasses in New Zealand, while of some benefit for livestock grazing, are increasingly valued as iconic grassland landscape species (Mark et al. 2013). The main areas of native grasslands are found in the central North Island, but more extensively in the South Island high country (Figure 2). There are 157 grass species endemic to New Zealand with an additional 31 indigenous species with a shared distribution in Australia and other Pacific Islands (Edgar and Connor 2010). These include silver tussock (Poa cita Edgar), fescue tussock (Festuca novae-zelandiae (Hack.) Cockayne), several Chionochloa spp. (e.g. C. rigida (Raoul) Zotov, C. rubra Zotov, C. flavescens Zotov and C. pallens Zotov) and Carex spp. (e.g. C. buchananii Bergg. and C. dipsacea Bergg.). Tussock grasslands deliver a wide range of important ecosystem services that provide many tangible benefits to human well-being but have generally not been quantified or evaluated (Mark et al. 2013). In addition, these biomes support endemic invertebrate communities that may be unable to adapt to modified grasslands and face displacement by exotic invaders, for example, 25 of New Zealand's 26 terrestrial amphipod species are endemic with evidence that they are being displaced in areas where an Australian invader, Arcitalitrus sylvaticus (Haswell) is present (Duncan 1994; Lowry and Myers 2019).

Based on 2018 data, New Zealand's agricultural production utilises 13.7 M ha (7.5 M ha in grassland and 2.4 M ha in native tussock or Rytidosperma), of which 8.8 M ha supports sheep and beef farming and 2.4 M ha dairy farming and 0.26 M ha deer farming (Anon 2020). Total agricultural exports in 2018-19 had an estimated value of NZ\$34.16 B (Free On Board (FOB)) of which pastoral exports were worth an estimated \$24.4 B (FOB) (Anon 2020). However, this is impacted, on average, by estimated losses to insect pests of NZ\$2.3 B p.a. (Ferguson et al. 2019).

Intensively developed New Zealand pastures consist of combinations of plant species predominantly based on a grass/legume mix. The most extensively used species are



**Figure 2.** Map of New Zealand, showing the geographic distribution of the grassland and crop, land use classes. Land use classes sourced from the New Zealand Land Cover Database (LCDB), using New Zealand's 1:50,000 topographic database (https://www.linz.govt.nz/land/maps/topographic-maps/topo50-maps). Reference: Thompson S, Gruner I, Gapare N (2003) New Zealand Land Cover Database Version 2: Illustrated Guide to Target Classes, Version 5.0\_January 2020, 126 pp.

perennial, short rotation hybrid and annual ryegrasses (*Lolium* spp.) and white clover. Other pasture species are tall fescue (*Schedonorus phoenix*), cocksfoot, timothy (*Phleum pratense*), red clover, chicory (*Chicorium intybus*) and plantain. In less developed pastures, browntop (*Agrostis capillaris* L.) is heavily utilised.

Alfalfa is an important dryland species used for grazing and stored winter forage. It is particularly valuable to farmers in environments where conventional ryegrasswhite clover plant species cannot persist (Avery et al. 2008; Moot 2012). Alfalfa is grown across 200,000 ha producing approximately 12 tonne/ha and additionally fixing 30 kgN/tonne of legume grown. Animal production from alfalfa is approximately 700 kg of red meat per ha (D. Moot, Lincoln University, pers. communication). Other pasture legumes also grown in New Zealand for dryland farming, but on a limited scale, are Caucasian clover (*T. ambiguum*) and subterranean (*T. subterraneum*) and balansa (*T. michelianum*) clovers (Moot 2012).

Supplementing grazing pastures, fodder brassicas (*Brassica rapa*, *B. napus* and *B. olearacea*), fodder beet (*Beta vulgaris*) and ryecorn (*Secale cereale*) are sown for specialised winter grazing, while maize is greatly utilised for silage. Cereals (wheat *Triticum* spp, barley (*Hordeum vulgare*) and oats, although predominantly grown as grain crops, are also produced for silage. The value of the main forage plant species to New Zealand is shown in Suppl. material 2: Table S2.

#### Insect pests and impacts

#### China

Much of the research on grassland pests has focused on the Inner Mongolian Plateau (Le et al. 2007) and, to a lesser extent, the Tibetan Plateau (Wang and Fu 2004). In Inner Mongolian native grasslands, the herbivorous insect species complex is dominated by 96 species of grasshoppers and locusts (Orthoptera: Acrididae) and these comprise the major pest group. The key pests are Oedaleus asiaticus Bey-Bienko, Calliptamus abbreviates Ikonn and Dasyhippus barbipes (Fischer-Waldheim) (Tu et al. 2015), especially on L. chinensis, oats and fescue in the Inner Mongolia, Xinjiang and river valleys in the eastern Qinghai-Tibet Plateau. In recent years, O. asiaticus has shown gregarious and migrating behaviour similar to that of the oriental migratory locust (Locusta migratoria manilensis (Meyen)) and has become one of the most damaging pests in northern China, at times accounting for over 90% of all grasshopper populations. On average, grasshoppers damage over 20 M ha of rangeland and forage crops p.a., consuming 1.6 M tonnes DM p.a. at an estimated cost of US\$80 M p.a. (Zhu 1999) (equivalent to US\$124 M in 2020 (813 M CNY or NZ\$172M)). Another assessment by Hong et al. (2014) estimated losses attributable to grassland locusts at 1.6 B CNY p.a. from 2003 to 2012. On the Qinghai-Tibetan Plateau, larvae of a species complex of the genus Gynaephora (Lepidioptera: Lymantridae) (Yuan et al. 2015), are significant pests of grasslands (Yan et al. 2006; Zhang and Yuan 2013). In 2003, an outbreak in the Qinghai Province was estimated to cover an area of 1 M ha<sup>2</sup>, leading to a loss of over

90 M CNY (Zhang and Yuan 2013). In addition, the presence of cocoons in the litter can cause skin irritations and blisters to livestock (Yan et al. 2006).

Larvae of several other Lepidoptera also damage grasslands. These include the beet webworm (or meadow moth) (*Loxostege sticticalis* (L.), the northern armyworm (*Mythimna separata* Walker and the fall armyworm (*Spodoptera frugiperda* (J.E. Smith). *Loxostege sticticalis* is a widely distributed, polyphagous, migratory species (Wei et al. 1987; Sun et al. 1995) that can cause significant losses in grasslands and forage crops during outbreak years (Hong et al. 2013; Tu et al. 2015). A list of key pest species in grassland and alfalfa in China can be found in Suppl. material 3: Table S3.

For alfalfa, the main pests are aphids and thrips, followed by alfalfa weevil (Hypera postica (Gyllenhal) (Coleoptera: Curculionidae), but published literature lists between 55 (Zhang et al. 2016) and 269 (Zhang et al. 2018) pest species, in a range of taxa and which attack all stages of the plant. Aphids and thrips are the most widespread taxa throughout the alfalfa growing regions, while *H. postica* is significant in the Ningxia and Xinjiang regions. Other pests include: Heliothis viriplaca (Hufnagel) (= Heliothis dipsacea) (He et al. 1997) and beet webworm (Loxostege sp.) (Zhang et al. 2005a). Both aphids and thrips reduce the yield and nutritional value of damaged alfalfa (Zhang et al. 2005b; Wu et al. 2013; Zhang et al. 2017) and also vector viral plant diseases. Aphids have been reported to transmit alfalfa mosaic virus (AMV), alfalfa leaf curl virus (ALCV) and bean mosaic virus (Garran and Gibbs 1982; Roumagnac et al. 2015; Ryckebusch et al. 2020) and recently two viruses (AMV and Medicago sativa alphapartitivirus 1 (MsAPV1)) have been identified in thrips (Li et al. 2021). The main aphid pest species are the blue alfalfa aphid (Acyrthosiphon kondoi Shinji), cowpea aphid (Aphis craccivora Koch), pea aphid (Acyrthosiphon pisum (Harris)) and spotted alfalfa aphid (Therioaphis trifolii (Monell)). The major thrips species is Odontothrips loti (Haliday) while Thrips tabaci Lindeman, Frankliniella occidentalis (Perg.) and Frankliniella intonsa (Trybom) also cause damage.

The area sown in alfalfa is expanding year by year and the most recent data showed that, in 2017, 4.15 M ha of alfalfa were being grown with a yield of 29.3 M tonnes (National Animal Husbandry Service 2017). As the area sown in alfalfa increases, so too do the incidence and impact of insect pests. Based on 2017 data, it is estimated that alfalfa pests cause at least 20% yield loss, with an average direct economic loss of 9.144 B CNY p.a (2.03 B NZD p.a.) (Li et al. 2020). Large scale outbreaks of *T. trifolii* have been estimated to cause economic losses to growers of between 0.151 to 1.127 B USD (Wu et al. 2013). Currently, *H. postica* is considered a major constraint on alfalfa production, as currently there are no effective control measures available (Zhang 2015).

#### New Zealand

In New Zealand tussock grasslands, severe damage to plants by indigenous insects is uncommon although occasionally observed, for example, grass grub (*Costelytra giveni* Coca-Abia & Romero-Samper), several species of the moth commonly called porina
(Wiseana spp.) and striped chafer beetle (Odontria striata White) (Campbell 1982; Barratt et al. 1988). Similarly, the impact of exotic insects within these systems seems low, although Argentine stem weevil (Listronotus bonariensis) Kuschel (Coleoptera: Curculionidae) (ASW) is widespread (Murray et al. 2003) and has been shown to feed on seedling blue tussock (Poa colensoi Hook.f.), F. novae-zelandiae and narrow-leaved snow tussock (Chionochloa rigida (Raoul) Zotov) and, potentially, could impact on seedling survival (Barratt et al. 2016). Indigenous insects have been shown to feed on exotic plants in areas where native vegetation is largely intact; for example, the native weevils Chalepistes spp. (formerly Irenimus spp., Brown 2017) and Nicaeana spp. (Barratt and Johnstone 1984; Barratt et al. 1992) will feed on and damage clover and seedlings. Similarly, the same genera can be common within cultivated and sown pastures consisting of entirely exotic pasture plants, but are not known to cause significant damage to these.

Insect pests are a persistent and significant economic cost to improved grassland and forage production systems in New Zealand (e.g. Zydenbos et al. 2011; Jackson et al. 2012; Ferguson et al. 2019), with a small number having a major impact on production and longevity of pastures (Suppl. material 4: Table S4). The major pests in improved grasslands are the endemic grass grub and porina, especially *W. cervinata* (Walker) and *W. copularis* (Meyrick) (Richards et al. 2017), along with the exotic ASW, clover root weevil (*Sitona obsoletus*, Gmelin) (CRW) and African black beetle (*Heteronychus arator*) (F.) (Ferguson et al. 2019). The estimated annual costs to agriculture are shown in Table 1.

Several lesser, or locally significant, pests also impact on production and persistence: black field cricket (*Teleogryllus commodus*) (Walker) (Orthoptera: Gryllidae), clover flea (*Sminthurus viridis*) (L.) (Collembola: Sminthuridae) and African black beetle are recurrent pests in the warmer areas of North Island, while Tasmanian grass grub (*Accrosidius tasmaniae* (Hope)) is widespread and locally damaging.

In part, the significant impact caused by endemic species, such as grass grub and porina, can be attributed to the high nitrogen and low fibre content of the exotic plant species compared to the native plant hosts (e.g. Atijegbe et al. 2020). Endemic scarabs that can have significant impacts locally include species from the genera *Odontria* (e.g. *O. striata* (White)) (Ferguson et al. 2019) and *Pyronota* (e.g. *P. festiva* F. and *P. setosa* (Given)) (Townsend et al. 2018). Endemic Orthopteran species, also found in improved grasslands, include the small field cricket (*Bobilla* spp.) and grasshopper (*Phaulacridium marginale*) (Walker). Neither is considered pests.

With the relatively recent increased use of the herb, narrow leaf plantain (*Plantago lanceolata*), in improved pasture (Stewart 1996), two previously insignificant, native Lepidoteran species *Scopula rubraria* (Doubleday) and *Epyaxa rosearia* (Doubleday) have emerged as pests, particularly when plantain is grown as a monoculture (Ferguson and Phillip 2014). Other pests that can specifically affect pasture seedling establishment include several lepidopteran species, including *Eudonia sabulosella* (Walker) and *E. submarginalis* (Walker) (both referred to as sod webworm) and *Agrotis ipsilon* (Hufnagel) (greasy cutworm). The wheat bug, *Nysius huttoni* White, is an endemic spe-

Insect species	Estimated cost (NZD M / CNY M)			Regions affected	
	Dairy		Sheep & Beef		
	NZD	CNY	NZD	CNY	
Grass grub (Costelytra giveni Coca-	156-425	736-2006	84-230	396-1086	All except northern North Island
Abia & Romero-Samper) *					
Porina (Wiseana spp.)	78–94	368 - 443	87–99	412-466	All except northern North Island
Black beetle (Heteronychus arator) (F.)	165-249	778-1177	17-21	79-101	Northern North Island some coastal areas
Argentine stem weevil ( <i>Listronotus bonariensis</i> , (Kuschel))	111–145	526 - 686	67–79	317-371	All
Clover root weevil ( <i>Sitona obsoletus</i> , Gmelin) **	68	321	195	920	All

**Table 1.** Summary of the mean annual monetary cost (NZD/CNY) of invertebrate pests to New Zealand agricultural production and the regions affected. Figures updated to 2020 costs from 2011 values<sup>1</sup>. M = Million.

<sup>1</sup> 2020 values determined from the % change in the New Zealand consumer price index 2011 to 2020. CNY values determined from CNY/NZD exchange rates as at 31 December 2020, 1 NZD = CNY 4.72.

\*includes mānuka beetle (Pyronota spp.) and striped chafer (Odontria striata, White).

\*\* cost after suppression through biocontrol by the parasitoid Microctonus aethiopoides.

Table adapted from Ferguson et al. (2019).

cies often found both in native and improved grasslands and is also an invasive species (Lay-Yee et al. 1997; Aukema et al. 2005). *Nysius huttoni* has a wide host range which includes *Triticum aestivum* (wheat), several *Brassica* and *Poaceae* spp., *M. sativa*, *T. repens* and *T. pratense* (EPPO 2006) and can have significant impacts on seedling survival (e.g. Gurr 1957; Ferguson 1994).

Alfalfa in New Zealand is grown either as a monoculture, destined to be harvested for stored winter feed and, to a lesser extent, for feed pellet production or as grazing alfalfa, either as a monoculture or in combination with grass, often tall fescue. It has only a few major pests, but the key species are all exotic and comprise *Sitona discoideus* Gyllenhal (in New Zealand called lucerne weevil) and three aphid species (*A. kondoi*, *A. pisum* and *T. trifolii*). The introduction of *A. kondoi* and *A. pisum* to New Zealand was also associated with an increase in the incidence of AMV in alfalfa stands (Forster et al. 1985). Lesser pests are white fringed weevil (*Naupactus leucoloma* (Boheman)) (King et al. 1982) and the little fringed weevil (*Atrichonotus taeniatulus* (Berg)) (Barratt et al. 1998).

#### Insect pest management

China and New Zealand face similar pest management issues. Traditional use of insecticides is no longer seen as the best option to mitigate pest impact and more focus is being placed on integrated pest management (IPM) systems utilising biopesticides, biological control and endophytes to mitigate pest impacts.

#### China

In grasslands, insects, entomopathogens and birds play an important role in naturally controlling pest populations. Insect biological control agents include dipteran species within Bombyliidae, Calliphoridae, Asilidae and Syrphidae, a range of coleopteran species of Carabidae (e.g. Tenebrionidae, Cicindelidae and Coccinellidae), neuropterans within the Chrysopidae and parasitoids. Entomopathogens include *Metarhizium anisopliae* (Metchnikoff) Sorokin, locust microsporidia (*Nosema locustae* Canning), *Beauveria bassiana* (Balsamo) Vuillemin, *Bacillus thuringiensis* Berliner and Entomo poxviruses (EPV). The former two are used extensively for grasshopper control, while *B. bassiana* and *B. thuringiensis* were mainly used for controlling soil pests, such as scarab larvae or lepidopteran larvae on foliage. These are usually applied to forage crops including maize and *M. sativa*.

Particularly in the control of locusts, pathogenic fungi and microsporidia are important in preventing the occurrence of outbreak populations. Strains of *M. anisopliae* and *B. bassiana*, specific to locusts, were introduced into China in 1990s and different formulations, such as an oil base, baits and wettable powder have been explored to optimise control in various grassland conditions. Since then, approximately 100 M hectares have been treated with *B. bassiana* (Zhang et al. 2000; Nong et al. 2007; Bian et al. 2009).

Locust microsporidium, isolated from *Locusta migratoria migratoriodies* (Reiche & Fairmaire) in the 1950s, has been shown to infect over 100 locust species and other orthopterans. It significantly reduces food intake, activity, fecundity and viability of eggs and causes insect death after 15 to 20 days (Wang and Yan 1999; Wu et al. 2000; Zhang and Yan 2008). Locust poxvirus is mainly used for locust control in Xinjiang, north-west of China, where *Calliptamus italicus* L., *Gomphocerus sibiricus sibiricus* (L.) and *Dociostaurus* spp. are the dominant locust species (Wang 1994).

Rosy starling (*Pastor roseus* L., formerly *Sturnus roseus*), hens (*Gallus gallus domesticus* L.) and ducks (Anatidae) are also used for localised control of grassland pests, especially grasshoppers and locusts (Lu et al. 2006). Rosy starlings have been shown to be an effective predator, with artificial nesting boxes used extensively to support populations (China Grassland Statistics 2018). The starlings feed both during the locust breeding and growth periods, with each adult starling shown to consume 120 to 180 locusts per day. Two studies carried out in the Xinjiang Region, showed that over a breeding season, rosy starlings were able to significantly decrease grasshopper densities (Guo 2005; Ji et al. 2008). Furthermore, starlings have been shown to follow locust swarms ensuring ongoing control (Guo 2005; Yu and Ji 2007). The use of poultry by farmers for pest control mainly occurs in Inner Mongolia and Xinjiang (China Grassland Statistics 2018).

A selection of insect biocontrol agents, both native and introduced and found in grassland and alfalfa in China, is shown in Table 2.

#### New Zealand

In natural New Zealand grasslands, several native natural enemies provide natural population regulation of significant endemic pests, particularly grass grub and porina. However, this breaks down in improved grasslands under agricultural management, primarily due to the disruption of soil-borne pathogens following cultivation. Some natural entomopathogens do regulate populations of these insects in older pastures,

Insect species	Host attacked	Pest stage attacked	Biocontrol agent origin
Coleoptera: Carabidae			
Lachnocrepis prolixa (Bates)	Various	Various	Native
Pterostichus gebleri Dejean	Various	Various	Native
Coleoptera: Cicindelidae			
Cicindela chinensis DeGeer	Various	Various	Native
Cicindela hybrida L.	Various	Various	Native
Coleoptera: Coccinellidae			
Coccinella septempunctata L.	Aphids	Adult and Nymphs	Native
Hippodamia variegata (Goeze)	Aphids	Adult and Nymphs	Native
Propylaea japonica (Thunberg)	Aphids	Adult and Nymphs	Native
Diptera: Syrphidae			
Chrysopa sinica Tjeder	Aphids	Adult and Nymphs	Native
Metasyrphus corollae F.	Aphids	Adult and Nymphs	Native
Hymenoptera: Braconidae			
Aphidius eadyi Starý, González & Hall	Acyrthosiphon kondoi Sinji	Nymphs	Native
Aphidius ervi Haliday	A. kondoi	Nymphs	Native
Aphidius eadyi	Acyrthosiphon pisum Harris	Nymphs	Native
Aphidius ervi	Acyrthosiphon pisum	Nymphs	Native
Aphidius picipes (Nees) (syn. A. avenea Haliday)	Sitobion avenae (Fabricius)	Various	Native
Hymenoptera: Eulophidae			
Chrysonotomyia trifolii	Coleophora frischella L.	Larvae	Native
Neuroptera: Chrysopidae			
Chrysoplera sinica (Tjeder)	Aphids	Adults and nymphs	Native

**Table 2.** List of insect biocontrol agents, both native and introduced found in grasslands and alfalfa inChina.

but they do not prevent pest outbreaks in young pastures (Jackson et al. 2012). Other natural enemies, for example, *Procissio cana* Hutton (Diptera: Tachinidae) which can parasitise up to 20% of third-instar grass grub larvae, are uncommon in highly modified pastures (Merton 1982). The introduction of several exotic biocontrol agents targeting these endemic pests failed (Cameron et al. 1989) and any similar attempts are now very unlikely to gain approval from New Zealand's Environmental Protection Authority.

Two endemic entomopathogenic bacteria, *S. proteamaculans* (Paine and Stansfield 1919), Grimont et al. 1978 and *Serratia entomophila* Grimont et al. 1988 (both Enterobacteriaceae), have been found to suppress larval populations of grass grub (Hurst et al. 2000; Hurst et al. 2018). The former is the only endemic biopesticide that has been commercially developed for use against pasture pests in New Zealand, but since its availability in 1990, uptake has been low. *Serratia proteamaculans* also has activity against mānuka beetle (*Pyronota* spp.) larvae. In addition, laboratory trials where mānuka beetle larvae were treated with the entomopathogenic fungi *Beauveria brongniartii* (Saccardo) Petch, produced high rates of larval mortality (Townsend et al. 2010), although formal field trials to determine efficacy have not been undertaken. Several porina-active microbial pathogens have been identified, including fungi, protozoa, viruses and nematodes (Bourner et al. 1996). The fungus, *M. anisopliae*, can cause infrequent epizootics of larvae (Latch and Kain 1983). The entomopathogenic bacterium, *Yersinia entomophaga* Hurst et al., 2011, isolated from grass grub has been shown in laboratory and field trials to have high levels of pathogenicity against larvae of two species of porina: *W. cervinata* and

*W. copularis* (Hurst et al. 2019). However, attempts to commercially produce formulations of indigenous pathogens, targeting these pests, are largely thwarted by the small market size and the relatively high cost of development (Glare and O'Callaghan 2019).

Three braconid parasitoids from the genus *Microctonus* have been introduced to provide biological control of three exotic weevil pests. *Microctonus aethiopoides* Loan (Moroccan ecotype) was released to control the alfalfa pest *S. discoideus* (Stufkens and Farrell 1980), *M. hyperodae* Loan for ASW (Goldson et al. 1992) and *M. aethiopoides* (Irish ecotype) for CRW (Gerard et al. 2006). All introductions were successful in suppressing damaging weevil populations, with both *M. aethiopoides* ecotypes providing natural control of what would otherwise have been major pests. An economic analysis of biological control of CRW in one region of New Zealand, found that control returned NZD14.78/ha/year and NZD6.86/ha/year for dairy and sheep & beef farms, respectively (Basse et al. 2015). Conversely, while *M. hyperodae* initially provided effective control of ASW (Barker and Addison 2006), recent evidence points to a breakdown in parasitoid efficiency and resurgence of weevil damage (Tomasetto et al. 2018).

The adult stage of *L. bonariensis* is susceptible to the entomopathogens *Microsporidium itiiti* Malone and *B. bassiana* (Barker et al. 1989), although they do not seem to provide effective control on the pest in the field. Isolates of *B. bassiana* have shown high levels of virulence against *S. obsoletus* (Nelson et al. 2015), but preliminary field trials showed limited success in the field (e.g. Brownbridge et al. 2006). Conversely, recent field trials of *Y. entomophaga* have demonstrated very good control of the adult stage of African black beetle (Mansfield et al. 2020).

An important strategy for protecting grasses (fescue and ryegrass) from insect pests in New Zealand is the development and use of fungal endophytes. Several *Epichloë* endophyte strains, producing different ranges of toxins that deter herbivorous insect feeding or oviposition (Schardl et al. 2013), are commercially available and their use is ubiquitous (Rodriguez et al. 2009; Schardl 2010). Such endophytes are effective in combatting ASW, African black beetle and porina, root aphids and pasture mealy bug (Popay and Hulme 2011) and show activity against grass grub (Ferguson et al. 2019).

Several biological control agents have been introduced and established in New Zealand for control of insect pests in improved grassland and alfalfa and are shown in Table 3. The list is compiled both from the Biological Control Agents Introduced to New Zealand (BCANZ) database, initially compiled from Cameron et al. (1989) and regularly updated as an online resource (Ferguson et al. 2007), along with published journal literature. It does not include predators, such as spiders (Vink et al. 2013), lacewings and ladybirds (e.g. *Coccinella undecimpunctata* L. (11 spotted ladybirds)), that may have some impact in improved grasslands, but which were not specifically introduced for that purpose. Feeding by the common starling (*Sturnus.vulgaris* L.), an exotic bird species introduced to New Zealand during European colonisation, has been shown in some instances to reduce grass grub populations by 40–60%, with predation able to reduce larval populations below levels that caused yield losses (East and Potting-er 1975). However, the authors concluded that the effectiveness of control depended on the presence of high starling numbers, with only localised control achievable.

Insect species	Host attacked	Pest stage attacked	Biocontrol agent origin	Reference
Hymenoptera: Braconidae				
Cotesia ruficrus Haliday	Agrotis ipsilon, (Hufnagel)	Larvae	Pakistan	Cameron et al. 1989
C. ruficrus	Chrysodeixis eriosoma, (Doubleday)	Larvae	Pakistan	Cameron et al. 1989
C. ruficrus	Mythimna separata Walker	Larvae	Pakistan	Cumber et al. 1977
Meteorus pulchricornis (Wesmael)	Lepidopteran larvae (polyphagous)	Larvae	Europe	Berry and Walker 2004
Microctonus aethiopoides Loan	Sitona discoideus Gyllenhal	Adults	Morocco	Stufkens et al. 1987
M. aethiopoides Loan	Sitona obsoletus (Gmelin)	Adults	Ireland	Gerard et al. 2006
M. hyperodae Loan	Listronotus bonariensis (Kuschel)	Adults	South America	Goldson et al. 1992
Aphidius eadyi Starý, González & Hall	Acyrthosiphon kondoi Sinji	Nymphs	Europe	Cameron and Walker 1989
A. eadyi	Acyrthosiphon pisum Harris	Nymphs	Europe	Cameron and Walker 1989
Aphidius ervi Haliday	A. kondoi	Nymphs	Europe	Cameron and Walker 1989
A. ervi	A. pisum	Nymphs	Europe	Cameron and Walker 1989
Bracon variegator Spinola	Coleophora frischella L.	Larvae	Germany	Pearson 1989
Hymenoptera: Eulophidae				
Chrysonotomyia trifolii Erdos	C. frischella	Larvae	Germany	Pearson 1989
<i>C. trifolii</i> Erdos	Coleophora mayrella (Hübner) syn. C. spissicornis	Larvae	Germany	Pearson 1989

**Table 3.** List of insect biocontrol agents introduced into grasslands and alfalfa in New Zealand. Introductions undertaken as part of classical biological control programmes.

## Discussion

International trade and tourism are seen as facilitating the global spread of invasive pests and plant diseases (Anderson et al. 2015; Early et al. 2016; Chapman et al. 2017; Evans et al. 2018; Seebens 2019) and associated loss of biodiversity (Paini et al. 2016; Courchamp et al. 2017; Li and Shen 2020). The threat posed by the movement of invasive species to China has been recognised (Xu et al. 2006; Wan and Yang 2016; Yan et al. 2017; Li and Shen 2020; Yu et al. 2020), with China's Belt and Road Initiative (BRI) being viewed as increasing that risk (Liu et al. 2019; Seebens 2019). Climate change also represents a threat, as a driver for changes in the impact of pests and diseases (Huo et al. 2012a, b; Gutierrez and Ponti 2014; Deutsch et al. 2018), providing new opportunities for range expansion into regions that hitherto were unfavourable (Bebber et al. 2013; Robinson et al. 2020) and influencing the efficacy and options of management strategies (Gutierrez and Ponti 2014; Robinson et al. 2020). As noted by Robinson et al. (2020), climate change will alter the nature of vectors and pathways, the abiotic nature of the recipient environment and the biotic interactions in recipient communities. While the respective size difference of China and New Zealand is considerable, with diverse climate and environments, there will be regions in both countries that share similar climates under current and future temperature predictions, as indicated in Kriticos (2012). Temperate grass species widely grown in New Zealand are also grown in parts of China (e.g. ryegrass, oats). Similarly, alfalfa is grown across a wide range of environments in both China and New Zealand. Forage species suitability mapping shows there are several regions in the south and east of China where ryegrass is and can be grown (Hannaway et al. 2005). Where similar grasses and forages are established, that situation provides a potential opportunity for establishment of exotic

insect pests. Examples include the alfalfa pests, S. discoideus which is established in New Zealand and H. postica which is established in China. If S. discoideus were to establish in China, or *H. postica* in New Zealand, both species would likely become high impact pests without effective control options. In this respect, climate matching for key pests will be critical to understanding potential risks (e.g. Kriticos 2011; Roigé and Phillips 2020). The impact of an invasive pest is also potentially high, as both countries are classified as global biodiversity conservation hotspots within BRI (Seebens 2019; Li and Shen 2020). An analysis of the cost of invasive insect pests and pathogens found that China could experience significant economic impacts from establishment (Paini et al. 2016). China already has several high impact exotic pests (Suppl. material 3: Table S3) and the recent arrival of S. frugiperda, via Africa, but originally from the Americas, is a pest with a wide host range (Wu et al. 2019). The establishment S. frugiperda also highlights the dynamic nature of biosecurity, as invasion routes are not always directly from country of origin, but that secondary invasion pathways can facilitate the global spread of unwanted pests (Mansfield et al. 2019). An example of anthropogenic long-distance dispersal was the interception of adult A. tasmaniae in Tuscany, Italy in a sea container that came from New Zealand (Mazza et al. 2014). Similarly, importation of forages into China from overseas suppliers is significant, including grass and alfalfa as hay, powder and granules (Guo et al. 2019). In 2016, China imported forages from USA (1.29 M tonnes), Australia (0.22 M tonnes), Mongolia (0.075 M tonnes) and Spain (0.072 M tonnes). Other minor sources were Canada, Argentina, Europe and Central Asia (Guo et al. 2019). Inadequate biosecurity protocols, or slippage at the border, potentially provide a pathway for invasive pests associated with these commodities. As emphasised by Early et al. (2018), rapidly changing global trade and transportation patterns open invasion routes that pest species rarely travelled in the past.

The highest economically valued grasslands in New Zealand are very simple ecosystems (Goldson et al. 2020), comprised of generally less than five sown exotic plant species. Considerable resources in the form of weed control, fertiliser inputs and management are expended to maintain these pastures in a highly productive state. The associated invertebrate communities are also viewed as simple (Goldson et al. 2014; Goldson et al. 2020) and, while a few indigenous insects have been able to adapt and exploit these habitats (e.g. C. giveni, Wiseana spp.), some niches remain unfilled and potentially vulnerable to invasion by exotic pests that arrive unaccompanied by natural enemies which, in their country of origin, would exert some natural population regulation. This has been demonstrated by the establishment and impact of the scarab beetle H. arator and weevils L. bonariensis and S. obsoletus (CRW). The latter exploits clover root nodules and roots. Clover roots are also subject to quite severe attack by the endemic grass grub, but this is usually transitory in younger pastures which have not built up naturally occurring pathogens after cultivation and sowing. Therefore, clover roots were a 'vacant' feeding niche until CRW invaded New Zealand and was able to exploit that niche, facing little competition from existing fauna. First observed in 1996 (Barratt et al. 1996), over the subsequent 18 years CRW colonised nearly all of New Zealand's agricultural land. Prior to the release of the biocontrol agent M. aethiopoides this led to a considerable reduction in clover survival and productivity. This resulted in increased costs to farmers to replace what was essentially a free nitrogen input and removing a facet of agriculture that allows New Zealand farmers to be internationally competitive. Another significant example of a vacant niche is alfalfa, currently largely free of invertebrate foliage feeders, but *H. postica*, could readily fill that niche should it arrive in New Zealand.

The 55 to 269 pests in alfalfa identified by Chinese researchers (Zhang et al. 2016; Zhang et al. 2018), substantially exceed those recognised in New Zealand (7). However, while biodiversity may be higher in China, it does not preclude significant impacts of invasive arthropods, such as has been shown with thrips *O. loti* in alfalfa (Zhang et al. 2017) and *S. frugiperda* in a range of crops (Wu et al. 2019).

Indigenous grasslands, while being more complex ecosystems, may also be threatened by new incursions. Displacement of indigenous invertebrates by exotic invaders is a reality, as evidenced by the Australian landhopper (*A. sylvaticus* Haswell), accidently introduced to New Zealand and displacing native Talitridae (Duncan 1994). The potential threat to indigenous insect species from an invasive species competing in the same habitat or detrimentally modifying the habitat is unknown, but could possibly be predicted, depending on the degree of knowledge around biology, plant hosts and climate similarities. The Chinese grassland invertebrate fauna is very different from New Zealand grassland fauna and contains several species that are recognised as pests in China (e.g. Acrididae (Suppl. material 3: Table S3)). Their impacts could be significant in New Zealand grassland systems. Similarly, the same could be said for selected high-impact pests currently found in New Zealand, but not China (Suppl. material 4: Table S4).

There is potential for significant impacts arising from pest incursions, but entomophagous biocontrol agents, already established in the respective countries, may provide a level of control to ameliorate losses. Kiran et al. (2019) assessed the potential of the resident exotic parasitoid wasp fauna, already established in New Zealand, to provide biotic resistance against possible future pests. They concluded that the current exotic species could potentially suppress 442 pest species not yet occurring in New Zealand. However, this approach may not always work, as has been shown with the Moroccan strain of *M. aethiopoides* against CRW (McNeill et al. 2000) or the relatively host specific parasitoid *M. hyperodae* (Barratt et al. 1997).

### Conclusions

While this review has identified some key grassland and alfalfa insect pests common to both New Zealand and China, there is a paucity of research that quantifies potential impacts on specific grassland or crops (e.g. alfalfa) in the respective countries. In addition, low impact pests in one country does not preclude their having a greater impact elsewhere, if they occupy underexploited niches, outcompete existing species or, through natural enemy release, have greater impacts on their plant hosts. While climate may limit range and impacts of an exotic species in the recipient country, field studies, whereby selected grassland species are established in climatically suitable regions in the country of origin,

provide the opportunity to identify and quantify pest impacts and address an important biosecurity issue. The sentinel plants concept, whereby plants growing in overseas locations can be used to identify and evaluate impacts of potential insect pest invaders, has proven to be valuable for identifying new pests and diseases for tree species (Vettraino et al. 2017; Eschen et al. 2019; Mansfield et al. 2019). Applying a similar approach to grassland and forage plants would seem logical for understanding impacts and providing information for pest risk analysis. From a New Zealand perspective, to remain internationally competitive grassland agriculture will rely on a limited number of high producing, but inherently vulnerable, plant species (e.g. ryegrasses, clovers and alfalfa). Any incursion and establishment of a pest able to utilise these plant species will threaten that competitiveness. Research that identifies potential high impact pests, prior to invasion and establishment, thereby provides early warning of pest risk. Understanding biology and behaviour also provides an opportunity to identify potential pathways by which a pest or pest complex could move between countries, as well as develop surveillance strategies for early detection in a recipient country. Furthermore, the sentinel plant concept allows for the identification of mitigation measures (e.g. plant resistance, biological control) that could be implemented in anticipation of an invasive pest's establishment in the recipient country.

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## Supplementary material I

# Table S1. The cultivated area and herbage yield of the main forage plant species in China

Authors: Mark R. McNeill, Xiongbing Tu, Colin M. Ferguson, Liping Ban, Scott Hardwick, Zhang Rong, Barbara IP Barratt, Zhang Zehua

Data type: species data

- Explanation note: Cultivated area data obtained from the China Forage Data Report 2016. Mu is the unit of area used in China, with 15 mu corresponding to 1 ha, M = Million.
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Link: https://doi.org/10.3897/neobiota.65.61991.suppl1

### Supplementary material 2

## Table S2. The predominant use and the monetary values (NZD/CNY) of the main forage plant species in New Zealand

Authors: Mark R. McNeill, Xiongbing Tu, Colin M. Ferguson, Liping Ban, Scott Hardwick, Zhang Rong, Barbara IP Barratt, Zhang Zehua

Data type: species data

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Link: https://doi.org/10.3897/neobiota.65.61991.suppl2

### Supplementary material 3

Table S3. List of main insect pest species found in grasslands and alfalfa in China Authors: Mark R. McNeill, Xiongbing Tu, Colin M. Ferguson, Liping Ban, Scott Hardwick, Zhang Rong, Barbara IP Barratt, Zhang Zehua

Data type: species data

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Link: https://doi.org/10.3897/neobiota.65.61991.suppl3

## Supplementary material 4

## Table S4. List of main insect pest species found in grasslands and alfalfa in New Zealand

Authors: Mark R. McNeill, Xiongbing Tu, Colin M. Ferguson, Liping Ban, Scott Hardwick, Zhang Rong, Barbara IP Barratt, Zhang Zehua

Data type: species data

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RESEARCH ARTICLE



## Classical biological control against insect pests in Europe, North Africa, and the Middle East: What influences its success?

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#### Abstract

Many factors can affect the success and failure of classical biological control. However, these factors have mainly been studied independently of each other, which leaves their relative importance within the complexity of classical biological control (CBC) programmes unknown. Therefore, we set out to take a more holistic view on the factors that may impact the outcome of CBC of insect pests by insect predators and parasitoids. To this end, we filtered the BIOCAT catalogue to extract entries for the Greater Western Palearctic ecozone and added 15 new explanatory variables. These mainly concerned traits of released biological control agents, target pests, and host plants of the target, but also included the number of introductions for specific agent-target combinations as a management aspect. We then analysed the data regarding three levels of success: agent establishment, impact on the target population, and complete control of the target. Between 1890 and 2010 a total of 780 introductions of insects for biological control were undertaken in the analysed area, constituting 416 agent-target combinations. Overall success of agent establishment was 32%, successful impact of single agents on their target was 18%, and success of complete control was 11%. The number of factors significantly influencing the outcome of CBC decreased with increasing level of success. Remarkably few agent-related factors influenced the success: insect predators as agents decreased the probability of establishment and using oligophagous parasitoids significantly decreased the chances of complete control. Other significant factors were related to traits of target pests or their host plants. For example, sap feeders and target pests attacking reproductive plant parts were more likely to be successfully controlled. The rate of success increased with the number of introductions of CBC agents, in particular against univoltine target pests. These findings suggest that a focus on agent-related traits to increase the chances of successful CBC is not fully justified and should be complemented with the consideration of lower trophic levels and other aspects of CBC, such as abiotic factors and management.

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#### **Keywords**

BIOCAT, importation biological control, introductions, invasive species

#### Introduction

Classical biological control (CBC), i.e. the introduction of natural enemies from the region of origin, has proven to be an efficient and cost-effective tool to control invasive insect pests worldwide, including pests of forests and ornamental trees (Van Driesche et al. 2008; Kenis et al. 2017). However, a global analysis of the trends in the CBC of insect pests by insect predators and parasitoids revealed that the success of introduced agents to control a target was only about 10% with only 29% of the target pests being satisfactorily controlled by at least one natural enemy (Cock et al. 2016). This estimation is surely conservative, because it includes many poorly designed CBC programmes and depends on the approach of estimation of the success (Heimpel and Mills 2017). Nevertheless, it raises questions as to why the success is not higher and what can be done to increase the success of CBC.

An impressive amount of theoretical and empirical evidence has been gathered to identify the specific factors that have an impact on the successful outcome of CBC programmes. They can be classified into five categories.

The first three categories are the biotic factors that are inherently bound to the three trophic levels involved in this type of biological control. They correspond to the life-history traits that are involved in the trophic interactions between the pest's host plant, the pest itself, and its introduced natural enemy. When considering which variables within these categories should be addressed to increase the success of CBC, the focus often falls on the highest trophic level, the selection of the biological control agent that is to be introduced. For example, it has been repeatedly suggested that parasitoids are more successful in target control, compared to predators (Hawkins et al. 1999; Kimberling 2004). Similarly, ectoparasitoids have been suggested to be less successful than endoparasitoids, although the reasons for that remain unclear (Stiling 1990), and parasitoids that kill hosts in earlier life stages other than eggs have a higher impact on target populations than those that kill later life stages, such as pupae (Mills 1994). Oligo- or polyphagy has also been suggested as a desirable trait for agents to survive periods when pest densities are low, although, to lower the risk of non-target attacks, the agents should be host-specific (Bale et al. 2008). Traits of lower trophic levels (i.e., target insects and their host plants) that may be related to the success or failure of CBC find, however, very little mention in the scientific literature. Bennett (1974) reviewed some criteria for the determination of suitable targets for biological control, but concluded that almost all pests warrant consideration for biological control. Interestingly, Gross et al. (2005) found that lower trophic level factors such as pest order can successfully predict outcomes in CBC of insect pests. And Kenis et al. (2017) deliver empirical evidence that success rates of agent establishment and target

control were higher in CBC projects targeting pests of woody plants than pests of other types of plants. A reason for this might be that being perennial, trees provide a more stable and predictable environment, when compared to herbaceous plants (e.g., annual plants or crops). Therefore, biological control agents have generally more time to establish and reproduce and might thus be more successful when released against pests of woody plants.

The fourth category of factors that can have an influence on the success of biological control introductions concerns abiotic factors of the environment where introductions are made. An obvious factor may be the existence of physical barriers to dispersal. However, the abiotic factor that is the most considered in relation to the success and failure of biological control is climate. It has been estimated that climate is responsible for about 35% of failures of natural enemy introductions against arthropods (Stiling 1993). Hence, climate-matching models are now widely used to more effectively search for biological control agents that are suited for the climatic conditions of the area of introduction (reviewed by Heimpel and Mills 2017).

The fifth category of factors potentially affecting the success of CBC involves the management of biological control programmes, and in particular release procedures. Some examples for these factors are the timing of release; release location; the quantity, quality, and life-stage of individuals that are released; the number of repeated introductions of an agent species against the same target; or the number of agent species that are released against a specific target (e.g., reviewed by Beirne 1985). However, surprisingly few tools have been developed that may help practitioners to take informed decisions on how to release CBC agents and, all too often, releases become a matter of trial and error.

From the above list of factors that can influence the success of biological control introductions, it becomes sufficiently clear that CBC is very complex and its success depends on proper methods and decisions at different steps of the process. Even if many individual factors have been shown to influence the outcome of introductions, their importance relative to all other factors remains unclear. The main objective of this study is therefore to analyse the relative importance of factors that possibly affect successes and failures of CBC of invasive pest insects using a holistic approach that reflects the complexity of biological control introductions. The analysis is done on the basis of the BIOCAT catalogue of biological introductions (Cock et al. 2016), which we enhanced by adding relatively easily available factors, mainly related to traits of agents, targets, and their host plants, but also one management-related factor. Furthermore, the analysis is restricted to available data from the Greater Western Palearctic ecozone (sensu Mitchell 2017; Suppl. material 1: Fig. S1), which includes Europe, North Africa, and the Middle East, on which we had a better knowledge and a better access to information sources, to increase the quality of the data researched rather than the quantity. The study is to be understood as an example of how the relative importance of factors from several aspects of CBC changes, when analysed in a more holistic context. Nevertheless, it may lead to suggested approaches for improving the success of future biological control introductions.

## Methods

### Data compilation

The basis for all analyses in this report is the BIOCAT catalogue of introductions of insects for the CBC of insect pests (Greathead and Greathead 1992; Cock et al. 2016). The simple spreadsheet database includes information from the published literature up until the year 2010 to provide some details of all biological control agents, target pests, introduction location and date, and the success of the introduction in terms of establishment and various levels of control. Data from 2011 on were not included because they were not yet completely updated at the time of the analysis. A detailed description of the database and an overview of trends in the use of insects for the CBC of insect pests is provided by Cock et al. (2016). For the present analyses, we filtered the database to extract entries for the Greater Western Palearctic ecozone (Suppl. material 1: Fig. S1) and added 15 new variables that were either populated based on existing variables in the database or based on information from published peer-reviewed scientific literature or available grey literature (i.e., reports). The new variables are described below and Table 1 provides an overview of the variables and their categories.

• Biological control agent feeding strategy: Either insect predator or parasitoid, based on the taxonomy and description of the species' life-history.

• Biological control agent host range: This variable was divided into four categories: monophagous (feeding on species within the same genus), oligophagous (feeding on species within the same family), polyphagous (feeding on species of several families or orders) or unknown (when no conclusive information about the host range was found). Information was retrieved from the scientific and grey literature. A valuable source for this variable and others related to the agent was Gerber and Schaffner (2016).

• Life-stage killed by the biological control agent: This variable was divided into five categories and populated from the scientific and grey literature. The categories were: eggs, larvae, larvae and adults, all stages, and other. The latter includes rare cases (<20) of more than one killed life-stages, such as eggs and adults, larvae and pupae, pupae, adults, and other combinations.

• Parasitoid feeding behaviour: This variable was created for parasitoids as agents only, which were categorised into endoparasitoids (feeding and developing within the body of their host) and ectoparasitoids (feeding and developing externally on the body of the host).

• Parasitoid brood size: This variable was created for parasitoids as agents only, which were categorised into solitary (only one offspring is ever produced per host) or gregarious (several offspring may be produced from a single host). As the brood size of a parasitoid species can vary depending on the host species or even host size, a parasitoid that has variable brood sizes was considered to be gregarious. The information was retrieved from the literature. In cases for which no information was found, the brood size was categorised as unknown.

• Parasitoid attack strategy: This variable was created for parasitoids as agents only, which were categorised into koinobionts (allow hosts to continue to grow in size after parasitism) and idiobionts (the host does not grow in size after parasitism and is often paralysed by the parasitoid). The information was retrieved from the literature. In cases for which no information was found, the attack strategy was categorised as unknown.

• Target pest feeding guild: Based on their relative abundance within different feeding guilds, targets were divided into three categories: borers (includes borers, gall inducers, miners, and tubers), defoliators, and sap feeders (mostly Hemiptera). The information was retrieved from the literature.

• Target pest host range: The classification was the same as for agent host range, but because of the relatively low frequency of mono- and oligophagous target species, only two categories were created: (1) mono- and oligophagous, and (2) polyphagous. The Crop Protection Compendium (2019) and the Invasive Species Compendium (2019) were the main sources to populate this variable.

• Target pest voltinism: Targets that only undergo one generation per year were categorised as being univoltine and those that undergo more than one generation per year as multivoltine. Targets that take more than one year to complete one generation were categorised as being univoltine, and those that may undergo one or several generations per year (e.g. based on climatic conditions) were categorised as being multivoltine. The information was retrieved from the literature.

• Host plant attacked by the target pest: The main host plants of the target insect were categorised as woody, herbaceous, or mixed (woody and herbaceous hosts). The information was mainly obtained from the Crop Protection Compendium (2019) and the Invasive Species Compendium (2019).

• Plant parts attacked by target pest: Based on the frequency of occurrence, targets were divided into four categories: shoot (stem and leaves), reproductive (flowers, fruits, seeds), both shoot and reproductive, and other (root, and other combinations).

• Number of introductions: For this variable, the database was collapsed to agent species-target species combinations and the number of introductions of one agent species against the same target species into the analysed area was counted.

• Successful establishment: This binomial variable was created based on BIO-CAT's "Impact code". A "0" was assigned to introductions that led to no, temporary, or not known establishment (impact code <1) and a "1" was assigned to introductions that led to permanent establishment (impact code  $\geq 1$ ).

• Successful impact: Also this binomial variable was created based on BIO-CAT's "Impact code". A "0" was assigned to introductions that led to no impact on the pest population (impact code <1.5) and a "1" was assigned to introductions that led to either partial control (reduced pest status but other control methods or agents are needed), substantial control (other control methods or agents are needed), or in small areas only), or complete control (no other control required) (impact code  $\geq$ 1.5).

• Complete control: As for successful establishment and impact, this binomial variable was created based on BIOCAT's "Impact code", with the difference that a "1"

was only assigned to introductions that led to complete control of the target (no other control required; impact code =2). All other cases (impact code <2) were assigned a "0".

Agent voltinism was not considered, because too few agents that were introduced into the analysed area were exclusively univoltine (n = 18).

#### Statistical analyses

Summary statistics were calculated with various Microsoft Excel functions using two modes: (1) Considering all introductions included in the database regardless if the same species was introduced once or several times against the same target; and (2) collapsing the database to unique combinations of an agent species introduced against a target species (henceforth called agent-target combinations) and keeping track of the number of introductions per unique species combination as a variable (see above), as well as calculating the sums of successful establishment, impact, and control for each combination separately. For the description of the trends over time, a decade is defined as a ten-year period and named based on their shared tens digit, from a year ending in a 0 to a year ending in a 9. For example, the period from 1960 to 1969 is the 1960s.

To assess the effect of the above described factors on (1) agent establishment, (2) impact of the agent on the target, and (3) complete control of the target, three separate Generalised Linear Mixed-Effects Models (GLMMs) with a binomial frequency distribution (logistic regressions) were conducted using the *glmmTMB* function of the package with the same name (Brooks et al. 2017) in R (R Core Team 2019). Success was used as dependent binomial variable and each full model included nine single factors and four interactions (see Table 1) as fixed effects, as well as random effects

Independent variable	Levels	Description	
Agent feeding strategy	2	predator, parasitoid	
Agent host range	4	mono-, oligo-, polyphagous, unknown	
Life-stage killed by the agent	5	eggs, larvae, larvae & adults, all stages, other	
Parasitoid feeding behaviour <sup>1</sup>	2	endoparasitoid, ectoparasitoid	
Parasitoid brood size <sup>1</sup>	3	solitary, gregarious, unknown	
Parasitoid attack strategy <sup>1</sup>	3	koinobiont, idiobiont, unknown	
Target feeding guild	3	borers, defoliators, sap feeders	
Target host range	2	(1) mono- and oligophagous, (2) polyphagous	
Target voltinism	2	univoltine, multivoltine	
Plant attacked by target	3	herbaceous, woody, both	
Plant parts attacked by target	4	shoot (stem and leaves), reproductive (flowers, fruits, seeds), shoot & reproductiv	
		other (root, and other combinations)	
Number of (No) introductions	Continuous	Number of introductions within agent-target combinations (1-34)	
№ Introductions × Agent feeding strategy	NA	Interaction	
№ Introductions × Target feeding guild	NA	Interaction	
№ Introductions × Target voltinism	NA	Interaction	
№ Introductions × Plant attacked by target	NA	Interaction	

**Table 1.** Independent variables used in the full models for CBC agent establishment, target impact (partial to complete control), and target control (no other control is needed).

<sup>1</sup> Only included as independent variable in models for parasitoids as biological control agents.

on agent and target species to account for correlations within agent-target combinations. The addition of more interaction terms as fixed effects led to problems with model convergence. A separate set of three logistic regressions was conducted for only parasitoids as agents, for which three additional factors were considered (Table 1). All models were tested for collinearity of fixed effects using the *check\_collinearity* function of the PERFORMANCE package (Lüdecke et al. 2020). Fixed effects that led to high correlation (variance inflation factor (VIF) >10) or moderate correlation (VIF >5<10) were removed sequentially, starting with the fixed effects displaying the highest VIF, until only low correlations (VIF<5) were present. All models with the remaining fixed effects were then tested for overdispersion of residuals using a Pearson chi-square test.

#### Results

#### Spatio-temporal trends of CBC

In Europe, North Africa, and the Middle East, the first recorded introduction of an insect as a biological control agent against another insect was done by Egypt in 1890 with the introduction of the coccinellid *Rodolia cardinalis* from Australia against the cottony cushion scale *Icerya purchasi* (Hemiptera: Margarodidae), an invasive species from Australasia damaging citrus. The introduction was successful in that the agent became established and led to a complete control of the target. From this first case until the year 2010, a total of 780 introductions of insects for biological control of insect pests were undertaken in the analysed area.

From the 1890s to the 1960s the number of introductions per decade steadily increased up to a maximum of 120 attempts (Fig. 1A). Only in the 1940s the number briefly recessed, coinciding with World War II. After a maximum of CBC attempts in the 1960s, the increasing use of widely available chemical insecticides can be linked to the lower number of CBC agent introductions in the following three decades. However, in the 2000s a steep decline followed, which brought the number of introductions down to only 20. This low number of introductions can be attributed to emerging concerns towards the use of CBC, especially regarding adverse nontarget effects on native biodiversity (Howarth 1983, 1991). Over the whole period, the majority of the introductions (83%; Fig. 1B) were done against pests of woody plants, and thus, the above-described trends are dominated by those introductions.

The success of the introductions varied over time, but in contrast to the number of introductions, it does not exhibit an equally clear trend (Fig. 1C). While establishment and impact remained relatively high until the 1940s, successful control steadily declined until the 1950s. The reasons for this remain unclear, but may be due to the fact that, at first, CBC was used against the most obvious targets, while it became more experimental after that. From the 1940s to the 1990s, successful establishment increased, while control and impact increased as well, but at a lower rate, starting in the 1950s and 1960s, respectively. From the 1980s and 1990s to the 2000s, a decline at all levels of success is



**Figure 1.** Number and success of classical biological control (CBC) introductions. Upper two panels **A** number of introductions of CBC agents per decade introduced in Europe, North Africa, and the Middle East against pests on (full line) all plants, (dashed line) woody plants, and (dotted line) herbaceous plants **B** total number of introductions for all target host plants, woody plants, and herbaceous plants (black bars) including repeated introductions of agents against the same target and (grey bars) for unique agent-target combinations. Note that some targets feed on both woody and herbaceous plants. Lower six panels: Percentage of successful biological control introductions (left side) per decade and (right side) overall, against pests on **C**, **D** all plants **E**, **F** woody plants, and **G**, **H** herbaceous plants. Success was measured in terms of (blue line) agent establishment, (purple line) partial to complete control of the target (i.e., impact), and (red line) substantial to complete control (i.e., control). Repeated introductions within specific agent-target combinations are included in the calculation of the percentages per decade and in full-coloured bars. Light-coloured bars indicate percentages based on unique agent-target combinations.

visible, which could possibly be attributed to the fact that in this period, the selection of a CBC agent became increasingly based on its specificity and less on its efficacy. The overall success of agent establishment was 41.8% (and 31.7% when excluding repeated introductions of same agent-target combinations and analysing the best possible outcome), 23.6% for impact on the target (18.0% without repeated introductions), and the overall success of complete target control was 12.1% (10.8% without repeated introductions) (Fig. 1D). When analysing the temporal trends of successful CBC against pests of woody and herbaceous plants separately, it becomes evident that also here the patterns of success for woody plants (Fig. 2E) followed closely the pattern of all introductions combined, while the ones for herbaceous plants were more erratic (Fig. 2G), due to the low number of introductions in some decades (Fig. 1A). The overall success of CBC was considerably higher when targeting pests of woody plants, compared to those of herbaceous plants (Fig. 1E-H): The success of establishment was more than twice as high for woody plants (47.2 vs. 19.5%), the impact on target pests was almost four times as high (27.5 vs. 6.9%), and the control of pests more than three times as high (14.2 vs. 4.4%). When including repeated introductions, the overall success of CBC of all pests together (Fig. 1D) and pests of woody plants (Fig. 1F) was always higher than for unique agent-target combinations. However, for pests of herbaceous plants (Fig. 1H), it was the opposite because of several unsuccessful repeated introductions, such as the two-spotted stink bug Perillus bioculatus (Hemiptera: Pentatomidae) that was introduced to Europe 19 times against the Colorado potato beetle Leptinotarsa decemlineata (Coleoptera: Chrysomelidae) without any successful establishment, impact, or control.

Eight countries were responsible for more than two thirds (70.5%) of all introductions: Israel (16.3%), Italy (14.0%), Former USSR (10.1%), France (7.3%), Greece (7.1%), Spain (6.0%), Egypt (5.3%), and Cyprus (4.4%). Within these countries, the percentage of complete target control was very variable, ranging from 19.1 in Spain to 5.5 in Israel (Table 2).

CBC attempts were conducted for a total of 416 agent-target combinations. From these combinations, 78% of the agents were parasitoids (Fig. 2A), within which endophagous, solitary, and koinobiont parasitoids dominated (Fig. 2D–F). The majority of agents were known to be either oligophagous (39%) or polyphagous (35%) and fewer are described as monophagous (13%). For 13% of the analysed targets, no information was found about their specificity (Fig. 2B). Biological control agents attacking larvae (36%) or larvae and adults (30%) were clearly predominant (Fig. 2C). Considering the targets, sap feeders, polyphagous, and multivoltine species dominated (Fig. 2G–I). Furthermore, 70% of the targets were pests of woody plants and the majority fed on shoots, or shoots and reproductive plant parts (Fig. 2J, K). In most cases (74%), only one introduction per agent-target combination was done, and in only 11 cases, the number of introductions was above eight (mean = 1.9; Fig. 2L).

CBC agents released in the Greater Western Palearctic ecozone for the described time period belonged to 33 insect families. Within the agent-target combinations, four



**Classes of independent variables** 

**Figure 2.** Relative frequency of independent variable classes related to (red bars) biological control agents, (blue bars) biological control targets, (green bars) host plants of targets, and (black bars) management **A** agent feeding strategy **B** agent host range **C** life-stage killed by the agent **D** parasitoid feeding behaviour **E** parasitoid brood size **F** parasitoid attack strategy **G** target feeding guild **H** target host range **I** target voltinism **J** plant attacked by target **K** plant parts attacked by target, and **L** number of introductions within agent-target combinations.

insect families made up 72% of all agents, with the three most abundant ones (Aphelinidae, Encyrtidae, and Coccinellidae) being agents attacking targets belonging to the insect order Hemiptera (Fig. 3A). When plotting the successful complete control against the relative abundance of agent families, the overall trend is positive, showing that the most often used agent families (especially Aphelinidae, Encyrtidae, and Coccinellidae) are also most successful. Only Braconidae stand out to contribute below average to target control (Fig. 3B). When considering the relationship between establishment and impact on target species, most agent families are very close to the positive regression line ( $R^2$ =.61) and only Ichneumonidae stand out, as none of the established agents of this family had a considerable impact on target populations (Fig. 3C).

With 36 insect families, the biological control targets were similarly diverse as the agents. However, the top 72% of targets comprised eight families, which is twice the amount of the top agent families. Not surprisingly, the six most abundant target fami-

**Table 2.** Countries in Europe, North Africa, and the Middle East and their respective number of introductions of biological control agents, agents established, targets impacted, and targets controlled. Target impact is defined as partial (reduced pest status) to complete (no other control needed) control and target control is defined as substantial (other control needed occasionally) to complete control. Percentages (numbers in parentheses) are calculated for all countries for which at least 10 introductions are recorded in the BIOCAT database.

Country	Number of introductions	Agents established	Targets impacted	Targets controlled
		Number (%)	Number (%)	Number (%)
Israel	127	43 (33.9)	15 (11.8)	7 (5.5)
Italy	109	48 (44.0)	30 (27.5)	20 (18.3)
Former USSR	79	20 (25.3)	12 (15.2)	6 (7.6)
France	57	24 (42.1)	16 (28.1)	4 (7.0)
Greece	55	20 (36.4)	12 (21.8)	8 (14.5)
Spain	47	30 (63.8)	25 (53.2)	9 (19.1)
Egypt	41	14 (34.1)	7 (17.1)	6 (14.6)
Cyprus	34	22 (64.7)	5 (14.7)	3 (8.8)
Former SFR Yugoslavia	21	0 (0.0)	0 (0.0)	0 (0.0)
Former Czechoslovakia	19	3 (15.8)	0 (0.0)	0 (0.0)
Turkey	17	10 (58.8)	7 (41.2)	2 (11.8)
United Kingdom	15	4 (26.7)	3 (20.0)	2 (13.3)
Germany	14	5 (35.7)	5 (35,7)	0 (0.0)
Morocco	13	8 (61.5)	3 (23.1)	2 (15.4)
Poland	13	1 (7.7)	1 (7.7)	0 (0.0)
France (Corsica)	11	10 (90.9)	8 (72.7)	3 (27.3)
Oman	11	8 (72.7)	2 (18 2)	2 (18 2)
Portugal	8	6	3	3
Switzerland	8	6	6	2
Georgia	8	6	6	2
Malta	6	4	4	3
Tunicio	6	5	4	2
Tumsia Tumsia	6	)	5	2
Ukraine	6	2	0	0
Iran D	5	1	0	0
Russia	5	1	l	0
Syria	>	5	0	0
Austria	4	3	l	1
Algeria	3	2	0	0
Belgium	3	1	1	1
Spain (Canary)	3	2	1	0
Czech Republic	2	2	0	0
Hungary	2	0	0	0
Ireland	2	2	2	2
Portugal (Azores)	2	0	0	0
Spain (Balearic)	2	2	1	0
Sweden	2	2	2	1
Yemen	2	1	0	0
Afghanistan	1	1	0	0
Azerbaijan	1	0	0	0
Croatia	1	0	0	0
Denmark	1	1	1	0
Greece (Crete)	1	1	1	1
Greece (Rhodes)	1	0	0	0
Italy (Cuneo)	1	0	0	0
Jordan	1	1	1	1
Lebanon	1	0	0	0
Portugal (Madeira)	1	0	0	0
Saudi Arabia	1	1	1	1
Slovenia	1	0	0	0
Uzbekistan	1	0	0	0
Grand Total	780	326 (41.8)	184 (23.6)	94 (12.1)



**Figure 3.** Descriptive statistics of (red) biological control agent families, (blue) biological control target families, and (green) plant groups. Upper row: Relative abundance of **A** agent and **D** target families, and **G** crops represented in introductions for biological control against insects in the analysed area (Europe, North Africa, and the Middle East). Middle row: Relationship between the relative abundance of **B** agents **E** targets, and **H** crops in all introductions and successful complete control of the target. Lower row: Relationship between the percentage of agent establishment and the percentage of partial to complete control (impact) of the target for the most abundant **C** agent and **F** target families, and **I** crops. Repeated introductions of agent-target combinations are not considered.

lies (Diaspididae, Pseudococcidae, Coccidae, Aleyrodidae, Tephritidae, and Aphididae) belong to the order Hemiptera, the insect order in which the above described majority of agent families are specialised (Fig. 3D). Given their relative abundance, the successful control of pests belonging to the families Aleyrodidae and Pseudococcidae is above average (Fig. 3E) and agents also have an above average impact on Pseudococcidae when considering their percentage of successful establishment (Fig. 3F).

Almost one third (31.7%) of crops attacked by the target pests were citrus, followed by olive (8.7%), potato (4.6%), mulberry (3.9%), various fruits (3.6%), various fruit trees (3.4%), and avocado (2.9%; Fig. 3G). A wide variety of other crops including woody and herbaceous plants were included into the category "other", which made up 33.2% of all crops attacked. In 8% of all cases no information about the attacked crop was found. Considering these relative abundances, the success of controlling mulberry pests was clearly above average, while none of the pests of other fruits and potato were completely controlled (Fig. 3H). Also, when considering the relationship between suc-
**Table 3.** Analysis of Deviance Tables (Type II Wald chi-square tests) for fixed effects of the logistic regressions testing the factors influencing biological control agent establishment, impact of the agent on the target species, and complete control of the target species, considering **A** all biological control agents (predators and parasitoids) and **B** parasitoids as biological control agents only.

Variable	Establishment			Impact			Control		
	$\chi^2$ -value	df	Р	$\chi^2$ -value	df	Р	$\chi^2$ -value	df	Р
(A) All agents (Predators and parasitoids)									
Number of (No) introductions	17.9290	1	<0.0001	12.5121	1	0.0004	0.7041	1	0.4014
Agent feeding strategy	6.3983	1	0.0114	1.6525	1	0.1986	0.1924	1	0.6609
Agent host range	2.8100	3	0.4219	4.0226	3	0.2590	5.3605	3	0.1472
Target feeding guild	17.3652	2	0.0002	8.9079	2	0.0116	7.6286	2	0.0221
Target host range	0.2938	1	0.5878	0.6057	1	0.4364	1.0514	1	0.3052
Target voltinism	0.4109	1	0.5215	0.1767	1	0.6743	0.0001	1	0.9915
Plant attacked by target	1.5464	2	0.4615	2.4780	2	0.2900	1.7736	2	0.4120
Plant parts attacked by target	12.5481	3	0.0057	0.5769	3	0.9017	0.6256	3	0.8906
№ Introductions × Agent feeding strategy	0.0556	1	0.9491	0.0556	1	0.8136	1.0702	1	0.3009
№ Introductions × Target voltinism	5.2701	1	0.0217	4.5016	1	0.0339	3.2968	1	0.0694
(B) Parasitoids only									
Number of introductions	11.9774	1	0.0005	9.0146	1	0.0027	0.0037	1	0.9518
Agent host range	2.1136	3	0.5492	5.6368	3	0.1307	8.9607	3	0.0298
Agent feeding behavior	0.0299	1	0.8627	0.0153	1	0.9017	0.1627	1	0.6867
Agent brood size	1.3036	2	0.5211	0.4971	2	0.7799	0.0119	2	0.9941
Agent's attack strategy	1.3689	2	0.5044	2.0629	2	0.3565	1.1576	2	0.5606
Target's life-stage killed by agent	1.7470	3	0.6265	NA	NA	NA	NA	NA	NA
Target feeding guild	6.4020	2	0.0407	12.7859	2	0.0017	11.6543	2	0.0029
Target host range	0.0110	1	0.9164	1.3492	1	0.2454	1.0008	1	0.3171
Target voltinism	0.7702	1	0.3802	1.9263	1	0.1652	1.3506	1	0.2452
Plant attacked by target	1.5567	2	0.4592	3.4705	2	0.1764	3.3887	2	0.1837
Plant parts attacked by target	5.9048	3	0.1163	0.6848	3	0.8768	0.3072	3	0.9587
№ Introductions × Target voltinism	4.8460	1	0.0277	4.9852	1	0.0256	4.4323	1	0.0353

cessful establishment of agents and their impact on targets, mulberry pests were clearly impacted above average (Fig. 3I). Introductions of CBC agents against pests of all forest trees together (e.g. eucalyptus, pine, fir, which are among others within the category "other") represented 11.5% of all introductions. From these, 14.6% led to agent establishment, 10.4% to impact on the agent, and 10.4% to complete agent control.

#### Main drivers of CBC success

The explanatory variables 'number of introductions  $\times$  plant attacked', 'number of introductions  $\times$  target feeding guild' and 'life-stage killed by the agent' were removed from all but one of the regressions due to high collinearity. The variable 'life-stage killed by the agent' remained in the regression analysing the success of establishment of parasitoids as CBC agents.

For all CBC agents together, the number of explanatory variables significantly influencing the outcome of CBC decreased with an increasing level of success, i.e. from establishment to impact to control (Table 3A). While for target control only the target's feeding guild was a significant variable, the impact of the agent was also significantly influenced by the number of introductions and the interaction of 'number of



**Figure 4.** Back-transformed (odds ratios) significant estimates and 95% confidence intervals from the logistic regression determining the factors negatively (<1, on the left of the dotted line) or positively (>1, on the right of the dotted line) influencing the success of biological control introductions, for (left panels) all biological control agents and (right panels) parasitoids as biological control agents. Success was measured as (upper panels (**A**, **B**)) establishment of the agent, (middle panels (**C**, **D**)) impact on the target (partial to complete control), or (lower panels (**E**, **F**)) substantial to complete control of the target. Estimates with numbers followed by an asterisk (\*) are statistically significant at  $\alpha$ =.05, those without an asterisk at  $\alpha$ =.1.

introductions × target voltinism', and agent establishment additionally by the agent's feeding strategy and the plant parts attacked by the target.

In contrast to models for all CBC agents together, for parasitoids the number of significant explanatory variables remained the same at all three levels of success (Table 3B). For all three models, the explanatory variables feeding guild and the interaction 'number of introductions × target voltinism' were significant. For agent establishment and impact on the target, the single variable of the number of introductions was also significant, but for target control the third significant variable was the agent's feeding behaviour.

The models' estimates for predators and parasitoids together revealed that keeping all other variables constant, the odds for agent establishment significantly increased with the number of introductions against specific agent-target combinations, when the target was a borer (endophagous) and when it was a sap feeder, as well as by an interaction of the number of introductions and the target being univoltine. But agent establishment significantly decreased when the agent was a predator, and when the target fed on reproductive plant parts (Fig. 4A; Suppl. material 1: Table S1). The only difference when analysing parasitoids as CBC agents alone was that borer as targets only had the tendency (.05> $\alpha$ <.10) to increase the success of establishment (Fig. 4B; Suppl. material 1: Table S2).



**Figure 5.** Model predictions for the influence of the interaction between number of introductions within agent-target combinations and target voltinism (red=univoltine, blue=multivoltine) on the probability of (upper panels) agent establishment, (middle panels) impact on target, and (lower panels) target control, by (left panels) all biological control agents and (right panels) only parasitoids as biological control agents.

The impact on targets was also positively influenced by the number of introductions against specific agent-target combinations, by sap feeders as targets, and by the interaction of the number of introductions and univoltine targets, but only had the tendency to be negatively influenced ( $.05>\alpha<.10$ ) when agents were oligophagous and targets univoltine (Fig. 4C; Suppl. material 1: Table S1). The latter two estimates significantly negatively influenced the impact of parasitoids on targets (Fig. 4D; Suppl. material 1: Table S2).

The odds to control a target significantly decreased when the agent was oligophagous but significantly increased with sap feeders as targets. It had also the tendency to be positively influenced by the interaction of 'number of introductions × univoltine targets' (Fig. 4E; Suppl. material 1: Table S1). However, for control by parasitoids, additionally univoltine targets significantly decreased the odds of target control but the interaction term 'number of introductions × univoltine targets' significantly increased it. Furthermore, there was a tendency of herbaceous host plants of target pests to decrease the odds of control (Fig. 4F; Suppl. material 1: Table S2).

A graphical analysis of the interaction between the number of introductions and target voltinism for each level of success separately (establishment, impact, and control) shows that for both univoltine and multivoltine targets the probability of success increased with the number of introductions. However, for univoltine species an asymptote at 100% probability of success was reached at 10–20 introductions, while for multivoltine species the relationship was rather linear, with a decreasingly steep slope at increasing levels of success (establishment>impact>control; Fig. 5). For parasitoids alone, the only difference is that there was more overlap in the confidence intervals at the level of agent establishment and less variability in the probability of agent control for multivoltine species at high numbers of introductions (Fig. 5).

## Discussion

The overall success of biological control introductions of insect predators and parasitoids against herbivorous insects in Europe, North Africa, and the Middle East is comparable to the success of CBC worldwide (Cock et al. 2016). The dominance of introductions against pests of woody plants, however, is markedly higher in the area analysed here (70%), when compared to the worldwide percentage (55%; Kenis et al. 2017). This can be attributed to the high number of introductions against pests on citrus, e.g. in Israel, Spain, and Italy.

Until the 1990s, it was common practice to collect several agent species in the area of origin of the target and release them with no or only a minimum of studies in the invasive range of the pest. Our analysis shows that in Europe, North Africa, and the Middle East these rather uninformed and hasty introductions led to a slight increase in agent establishment but less frequently to a sufficient impact on the target. Furthermore, these introductions were often only attempted once for any given agent species, which can explain the high percentage of one-time introductions for agent-target combinations (74%). In fact, as has also been shown in a global analysis of the BIOCAT catalogue (Cock et al. 2016), the percentage of success in terms of establishment, impact on the target or target control, seems to be independent of the number of species introduced for the biological control of a given pest. However, it seems undeniable that the period of uninformed introductions decreased the overall percentage of success, when compared to the time between the 1900s and 1940s. Concerns of undesirable non-target effects beginning in the 1980s (Howarth 1983, 1991) and the resulting focus on specificity and less on efficacy of CBC agents, led to a further decrease of success until the 2000s. Thus, within one century of CBC in Europe, North Africa, and the Middle East, no overall increase in success was achieved, indicating that new ideas are needed about how to increase the success of CBC against insect pests.

On the other hand, we show here that for the agent-target combinations for which introductions have been repeated several times, a positive correlation between the number of introductions and success was found. There are several possible reasons why repeated introductions of the same agent against one target may lead to a higher probability of success. For example, if several introductions were made from different source locations, the more diverse genetic sources of the agents may lead to a higher possibility that at least one of the strains can well adapt to the new environment in the location of release (Hopper et al. 1993; Hoddle et al. 2015; Leung et al. 2020). On the other hand, a biological control agent might also have been introduced against the same target in different countries within the analysed ecozone, increasing the chance of finding a suitable habitat or climate in at least one of them. While the BIOCAT catalogue offers information on the source and release country for the agents, it is a tedious work to investigate the pathways of agents once they were introduced the first time into an ecozone. It might be possible to do it for some case studies but a detailed analysis of these possible mechanisms is beyond the scope of this study. There might also be a bias towards higher success with increasing numbers of introductions, i.e., when an agent from one source location was introduced into several locations because it already successfully controlled a target somewhere else. An example from our analysis are parasitoids of the family Aphelinidae, which once proven successful in aphid control, have been introduced more often and increased the percentage of successful control. Lastly, it is also possible that there is simply a positive learning curve with the number of introductions, so that success was warranted after a couple of failures from which the biological control practitioners could learn.

Regardless of the underlying mechanisms discussed above, we found that as few as 10 introductions increased the mean probability of agent establishment to 75% for univoltine species, and with 20 introductions success of CBC increased on all levels (establishment, impact, and control) to near 100%. However, those results were biased by the facts that in only 2.6% of agent-target combinations more than 10 introductions have actually been done, and that only 15.4% of targets were univoltine species. Nevertheless, it emphasizes the importance of the number of introductions for specific agent-target combinations for the success of CBC.

Interestingly, remarkably few agent-related factors significantly influenced the success of CBC. The odds of establishment decreased when agents were predators, a finding that has been repeatedly confirmed empirically by comparing the success of predators and parasitoids used in CBC programmes (e.g., Hawkins et al. 1999; Kimberling 2004; MacQuarrie et al. 2016; Kenis et al. 2017). The higher success rate of parasitoids

as well as the fact that they are generally more specific to their target than predators have led to them being used more often in CBC (Greathead and Greathead 1992; MacQuarrie et al. 2016; Kenis et al. 2017).

Furthermore, oligophagy of agents was negatively related to the impact and control of targets, which holds true when considering all CBC agents together or only parasitoids. Considering agent host range as a single factor, the overall success of target control was 21%, 8% and 10% (or 28%, 7%, 11% when considering repeated introductions) for mono-, oligo-, and polyphagous agents, respectively, which would suggest that rather monophagy is an advantageous characteristic over oligo- and polyphagy. Given the relatively low number of agents that were considered monophagous in this study (13.5%), the effect of agent host range on the success of CBC should be further assessed in a multiple regression context including data from additional regions of the world. Interestingly, oligo- and polyphagy have been mentioned in the literature as a desirable trait for CBC agents, e.g., to survive periods when target pest densities are low by switching to other prey/hosts (Bale et al. 2008). Nowadays, an insect's high degree of specificity to the target species is often a requirement for the import of a species as a CBC agent to prevent negative impacts on non-target species. However, host specificity can only be determined through extensive laboratory studies or field surveys before and after the introduction of a species. In the past, those studies were rather uncommon. While pre-release host specificity assessments are now routinely done in new CBC programmes, post-release monitoring studies are still not common (Van Driesche et al. 2008; Hajek et al. 2016). Therefore, it can be assumed that we overestimate host specificity in many cases, especially when relying on information from older studies, where no or few investigations of this CBC agent trait were done.

The factor with the strongest influence on the chances of success of CBC was related to the trophic guild of the target insect: sap feeders were the target feeding guild most likely to be successfully controlled. This result is consistent in our analyses through all levels of success and for both parasitoids and all agents combined. It is also consistent with results from previous analyses, where 'Homoptera' were repeatedly found to be the group of insect pests with the highest number of agent releases, establishments, and successful control (Hall and Ehler 1979; Hokkanen and Sailer 1985; Stiling 1990; Greathead and Greathead 1992). Also when considering specifically CBC attempts against insect pests of trees, the current suborder Sternorrhyncha was found to be targeted most often and with the highest percentage of success (Kenis et al. 2017). Examples for this in the region analysed here are (1) the eucalyptus psyllid Ctenarytaina eucalypti (Hemiptera: Psyllidae) that was targeted four times in Europe (France, Ireland and the UK) by the Australian parasitoid Psyllaephagus pilosus (Hymenoptera: Encyrtidae), each time leading to a complete control of the pest (Hodkinson 1999); and (2) the cottony cushion scale *Icerya purchase* (Hemiptera: Monophlebidae), which attacks citrus trees and was targeted 13 times (each time in a different country of the Greater Palearctic Ecozone) by the cardinal ladybird *Rodolia cardinalis* (Coleoptera: Coccinellidae), resulting in 9 cases (69%) of the introductions to complete control of the target pest (Greathead 1976). Also in a global analysis of the BIOCAT catalogue,

Heimpel and Mills (2017) found success rates against sap sucking hemipteran insect families (Aleyrodidae and Aphididae) to be the highest. As possible reasons for this higher success, Mills (2006) suggested that compared to parasitoids of lepidopterans, parasitoids of "homopterans" have more frequently multiple generations per host generation and typically a broader range of host stages available for attack. However, one has also to consider a possible sampling bias in our analysis as 6 of the 10 most targeted insect families were sap feeders, the majority of which are pests of citrus.

Our analysis only comprises a limited number of factors from the various aspects of CBC that have been reviewed so far. Many other factors may significantly influence the outcome of biological control introductions. In our opinion, especially climate matching and management factors such as the timing, the quantity, and quality of CBC agents being released deserve more attention to increase the success of CBC programmes. However, for the majority of the introductions that have been done, the principal challenge might be the limited availability of information about many of the potentially important factors explaining their success or failure. To further advance in this direction, more data have to be gathered, for which a more rigorous documentation of CBC programmes and a wider availability of these data to scientists and biological control practitioners are paramount. Additionally, our analysis was restricted to data from Europe, North Africa, and the Middle East. Because results may change among ecozones, BIOCAT data from other ecozones or for the whole world should also be analysed.

# Conclusions

The finding that only few CBC agent-related factors significantly influenced the success of CBC suggests that the reoccurring focus on agent-related traits is not justified and should be redirected to include lower trophic levels and other aspects of CBC, such as abiotic factors (i.e., climate) and management (e.g., release procedures). Indeed, Gross et al. (2005) already demonstrated that factors related to biological control targets and their host plants can effectively help to predict the success of introductions. Our study confirms this finding and identified factors of different aspects (agent, target, target host plant, and management) that significantly affect CBC at several levels of success: (1) Predators as CBC agents decreases the chances of establishment and oligophagous agents decrease the chances of impact and control; (2) sap feeders as targets increase the chance of success at all levels, borers as targets increase chances of agent establishment, and univoltine targets decrease the chances of impact and control; (3) when reproductive plant parts are attacked by the target the chances of agent establishment decrease; and (4) increasing numbers of introductions of the same agent against a specific target increases the chances of agent establishment and impact on targets. The latter also extends to target control by parasitoids when targets are univoltine. A summary of the results for woody plants can be found in the supplements (Suppl. material 1: Fig. S2).

The results from this study should be understood as a first step to give the incentive for a holistic, rather than an independent consideration of factors affecting the success of CBC. The analysis of the entire BIOCAT catalogue or an updated version including the more recent introductions should lead to further insights and help to develop decision support tools to increase the success of CBC at all levels.

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## Supplementary material I

#### Supplementary tables and figures

Authors: M. Lukas Seehausen, Catarina Afonso, Hervé Jactel, Marc Kenis Data type: Tables and figures

- Explanation note: **Table S1.** Model coefficients (estimate ± standard error) and their level of significance in the logistic regressions for all biological control agents (predators and parasitoids) analysing variables impacting agent establishment, agent's impact on the target, and target control. **Table S2.** Model coefficients (estimate ± standard error) and their level of significance in the logistic regressions for parasitoids as biological control agents, analysing variables impacting agent establishment, agent's impact on the target, and target control. **Figure S1.** The Greater Western Palearctic ecozone (sensu Mitchell 2017), comprising Europe, Northern Africa, and the Middle East, for which area the BIOCAT data were analysed here. **Figure S2.** Summary of the results for woody plants, depicting the percentages of overall success of biological control (red circles) for pests of woody plants with borers, defoliators, or sap feeders as target pests; predators or parasitoids as classical biological control (CBC) agents; and (black) single or multiple releases of an agent against the same target.
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