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Prioritising potential incursions for contingency planning: pathways, species, and sites in Durban (eThekwini), South Africa as an example

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Abstract

Increased trade and travel have resulted in an increasing rate of introduction of biological organisms to new regions. Urban environments, such as cities, are hubs for human activities facilitating the introduction of alien species. Additionally, cities are susceptible to invading organisms as a result of the highly altered and transformed nature of these environments. Despite best efforts at prevention, new incursions of alien species will occur; therefore, prioritising incursion response efforts is essential. This study explores these ideas to identify priorities for strategic prevention planning in a South African city, Durban (eThekwini), by combining data from alien species watch lists, environmental criteria, and the pathways which facilitate the introduction of alien species in the city. Three species (with known adverse impacts elsewhere in the world) were identified as highly likely to be introduced and established in Durban (*Alternanthera philoxeroides*, *Lithobates catesbeianus* and *Solenopsis invicta*). These species are most likely to enter at either the Durban Harbour; pet and aquarium stores; or plant nurseries and garden centres – therefore active surveillance should target these sites as well as adjacent major river systems and infrastructure. We suggest that the integrated approach (species, pathways, and sites) demonstrated in this study will help prioritise resources to detect the most likely and damaging future incursions of alien species.

Keywords

biological invasions, early detection, incursion response planning, prioritisation, alligator weed, southern sandbur, American bullfrog, red imported fire ant

Introduction

Human-related activities such as trade and travel have facilitated the increased introduction of biological organisms outside their native range (Hulme 2009, Tatem 2009, Faulkner et al. 2016a, Hill et al. 2016). Introduction of alien species (sensu Richardson et al. 2000) to regions outside their native range is a serious problem which can result in the loss of biodiversity, and have negative economic and social impacts (Lövei 1997, Pimentel et al. 2001, Kenis et al. 2009, Vilà et al. 2010, Vilà et al. 2011). However, not all alien species pose an unacceptable risk of becoming invasive and many have significant benefits. Moreover, the capacity to respond to the threat of biological invasions is limited, severely so in some cases (Early et al. 2016). It is thus impractical and even undesirable to prevent every alien species from being introduced into a new region. For these reasons, efforts to prevent biological invasions need to be prioritised.

McGeoch et al. (2016) suggest that prioritisation should incorporate three aspects – species, pathways, and sites. Specifically for prevention, priority should be given to species posing the greatest risk of invading new regions, the pathways facilitating their introduction, and sites most at risk of being invaded. For example, species can be assigned to watch lists based on pre-border risk assessments that inform prevention strategies and contingency plans (Genovesi and Shine 2004; Faulkner et al. 2014, Nehring and Klingenstein 2008, Parrot et al. 2009). The German-Austrian Blacklist System (GABLIS), one such example, assigns species to three different categories based on risk assessments: 1) species that are of concern and for which specific intervention is required; 2) species whose risk to biodiversity cannot be ascertained; and 3) species with no risk to biodiversity that can be imported (Essl et al. 2011). GABLIS is a fairly rapid and effective assessment of different taxonomic groups in a variety of environments and illustrates the benefits of using watch lists as an early warning system (Essl et al. 2011, Verbrugge et al. 2010). Similar approaches have been implemented in Germany ('warn list' for aquatic alien species – Nehring and Klingenstein 2008), Belgium (Branquart 2007) and South Africa (NEMBA prohibited species list – DEA, 2016; watch list of alien species – Faulkner et al. 2014).

Similarly, pathways facilitating the introduction of alien species to new regions need to be identified and the risk associated with introductions facilitated through these pathways assessed. Priority should then be given to the pathways of introduction which pose the highest risk of facilitating the introduction of alien species (Padayachee et al. 2017, Pergl et al. 2017). The aim of this approach is to reduce colonisation pressure (i.e., the number of alien species) and propagule pressure (i.e., the number of individuals of a given alien species) facilitated through high risk pathways of introduction (Hulme et al. 2008, Reaser et al. 2008). This approach is significant in targeting the prevention of multiple taxa being introduced to a variety of environments, and especially in responding to the unintentional introduction of alien species.

Finally, sites are assessed as high-risk based on the likelihood of an invasion (i.e., the exposure to incursions and whether incursions will establish themselves and become invasions) and sensitivity (i.e., most vulnerable to the impacts of invasions) (Wilson et al. 2017). Sites which are most at risk of being invaded and most sensitive to the impacts of invasions are given priority for targeting the surveillance of new alien species. An important consideration in prioritising sites for prevention efforts is to identify where species are likely to first be introduced and established. In this context, and given the preponderance of introduction pathways, it is important that some biosecurity efforts explicitly focus on cities. Cities can be considered as sites where invasions are likely to occur as a result of the high environmental heterogeneity, high transport intensity and high levels of disturbance present in these environments (Cadotte et al. 2017; Gaertner et al. 2017; Kowarik 2011; Kuhman et al. 2010; Pyšek et al. 2010). Moreover, cities are potentially sensitive if the impacts affect ecosystem services or humans directly (Hansen and Clevenger 2005; Potgieter et al. 2017). They are also often areas where there are many complex competing demands on natural resource managers [e.g. (Dickie et al. 2014) and for South Africa see (Gaertner et al. 2016; Irlich et al. 2017; Zengeya et al. 2017)].

In this study we identify potential future incursions in Durban (eThekwini), South Africa, based on selected alien species, the pathways facilitating their introduction, and the sites most at risk of being invaded by these species. By jointly considering species, pathways, and sites, we aim to provide a tool for decision makers to more effectively target surveillance and contingency planning.

Methods

The eThekwini municipality is one of the largest port cities on the east coast of the African continent and is an important economic centre in South Africa (Roberts 2008). In addition to being a major populated city (approximately 3.4 million – STATSSA, 2017), eThekwini is also a significant contributor to tourism (Roberts 2008). Resources to target the introduction of alien species are scarce; therefore prioritisation is essential to effectively respond to the introduction of alien species.

To develop a methodology for decision makers to assign priorities for prevention strategies we: 1) identified cities with a similar climate to eThekwini; 2) used existing lists of species considered as not present in South Africa that pose an unacceptable risk of invasion; 3) identified which of the selected species are likely to have pathways facilitating their introduction to eThekwini; 4) developed climatic suitability models for the selected species based on the climate in eThekwini; and 5) linked the climate and pathway information to identify sites within eThekwini that should be the focus of contingency planning for particular species (Figure 1).

Human population, as a result of the associated activities (trade and travel), is one of the main correlates of species introductions into regions outside of their native range (Hulme 2009, Carpio et al. 2016), while climate is one of the main limitations to species establishment in these new regions (Rejmánek and Richardson 1996, Welk et al. 2002, Robertson et al. 2004, Thuiller et al. 2006). The methodology used in this study is required to be easily implementable and adjustable to various urban con-

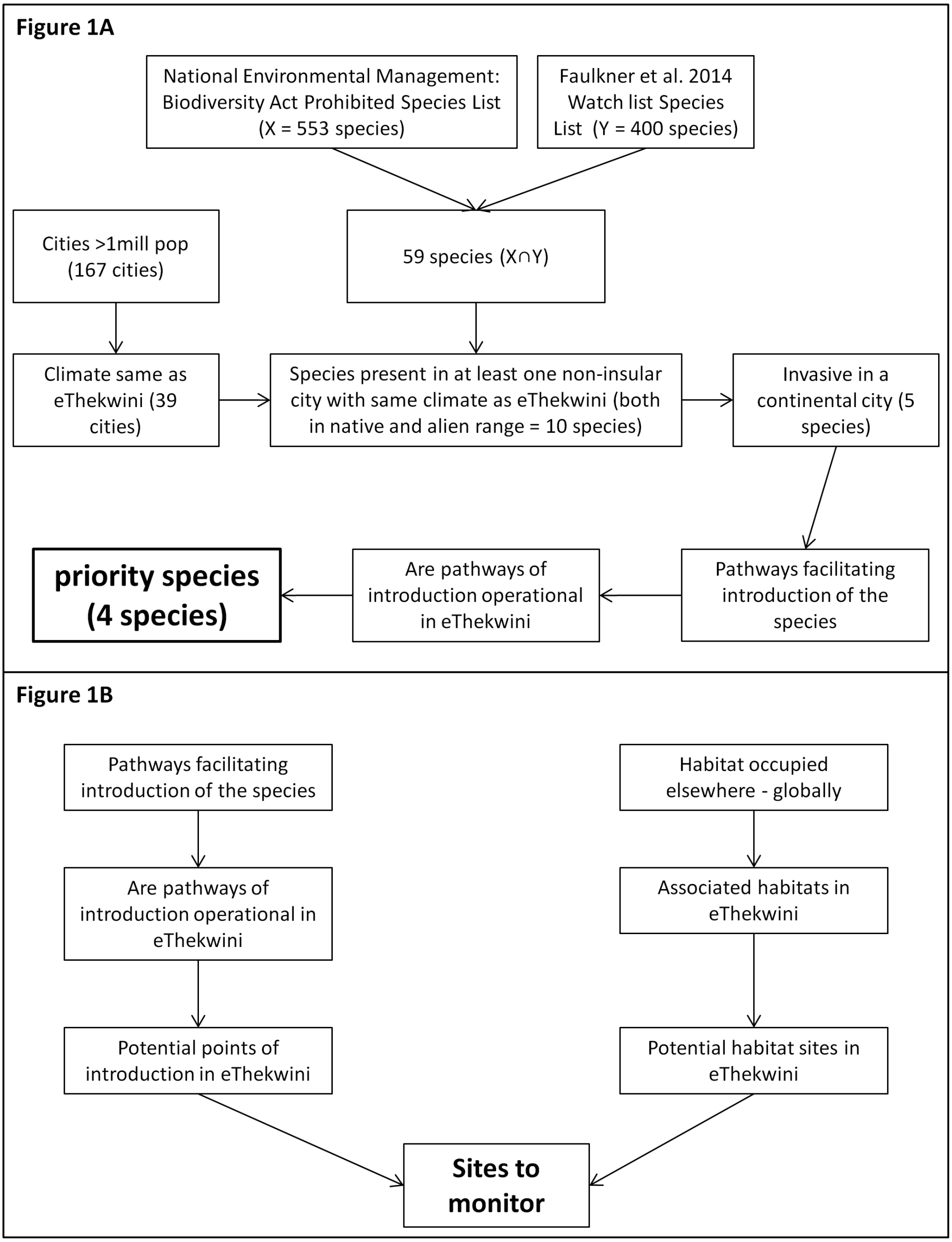

Figure 1. A simple and rapid method to prioritise targets for contingency planning to prevent biological invasions. The method identifies priority sites for managing particular high-risk incursions. **A** Shows the selection criteria used to select target species for climatic suitability analyses, with the number of species selected at each stage of selection indicated in parentheses. **B** Shows the criteria used to identify potential points of introduction for the select target species, as well as the criteria used to identify potential points of naturalisation, i.e. priority sites for monitoring in the eThekwini municipality.

texts; therefore we considered cities across countries with varying economic statuses. We selected global cities with populations of >1 million people (Padayachee et al. 2017) and used climate-matching techniques to select cities, from this list, with the same climate type as eThekwini based on the Köppen-Geiger climate classification (Kottek et al. 2006).

The National Environmental Management: Biodiversity Act (No. 10 of 2004) governs all biodiversity related issues in South Africa, including biological invasions (NEMBA, 2014). In regulations under NEMBA, a prohibited species list was created, based in part on expert opinion, that lists species that are not believed to be present in South Africa and whose introduction should be prevented (DEA, 2016). The implication is that strategic prevention plans should be developed for all species on the prohibited list. Separate to this, Faulkner et al. (2014) created a watch list of alien species whose introduction into South Africa should be regulated (based on likelihood of introduction, likelihood of establishment, and impact elsewhere). In this study we considered species present on both of these lists, as these are species that have been identified as high-risk and the regulations mandate government entities (e.g. municipalities) to manage such species.

We used these national lists and applied our own selection criteria (Figure 1) to identify species which should be prioritised for eThekwini. We ascertained the native and alien range of species using the CABI Invasive Species Compendium database (CABI 2017 – https://www.cabi.org/isc/) and the Global Register of Introduced and Invasive Species database (GRIIS 2017 – http://www.griis.org/). We downloaded occurrence data for all the species in both their native and alien range from the Global Biodiversity Information Facility (GBIF 2017a, b, c, d). Species occurrences for which sources were not listed, or were listed as "unknown" in the GBIF database, were removed from the dataset; additionally (for plant species) we removed occurrences based on herbarium records. Species with inconsistent taxonomic classification were also excluded (i.e., species for which variations and subspecies were only listed in GBIF). The occurrence records were then mapped and converted to shapefiles using ESRI ArcMap 10.3.1 software (ESRI 2015). Species occurrence records were then overlaid on to the selected cities. Species which occurred within the topographical boundaries of cities with the same climate as eThekwini were selected (regardless of whether the species were native or alien to the city). Furthermore, we excluded species which were only found as alien on islands (including Australia). This was on the assumption that biotic resistance is different on islands and continents. We then selected species present (as either native or alien) in cities with the same climate as eThekwini. We used the CABI Invasive Species Compendium (CABI 2018 – https://www.cabi.org/isc/) and Global Invasive Species Database (GISD 2018 – http://www.iucngisd.org/gisd/) to identify the pathways facilitating the introduction of the remaining species to see if they might be introduced to eThekwini. The description of the pathways used in this study was as per the Convention of Biological Diversity pathway classification scheme (Harrower et al. 2017; Hulme et al. 2008; Scalera et al. 2016) .

Maximum entropy distribution modelling was selected to map the potential geographic distribution and evaluate the risk of invasion of the remaining species (Maxent v3.4.1 – Phillips et al. 2006, Phillips et al. 2008). Even though Maxent has limitations in its representation as being a "presence-only data" algorithm, the software by default selects pseudo-absences in the form of background data and hence works well for presence-only datasets, such as the datasets downloaded from GBIF and used in this study (Barbet-Massin et al. 2012). Furthermore, predictions are robust as small sample sizes and irregularly sampled data do not strongly affect the model produced (Pearson et al. 2007, Elith et al. 2011). We chose to primarily utilise the default settings used by Maxent: 1) 10 000 random background points were assumed to be pseudo-absences points, however, we restricted the selection of background points to select points from the species distribution range (native and alien); 2) create response curves to evaluate the species response to individual predictors; 3) use a logistic output to produce continuous maps and 4) perform a jack-knife procedure to assess individual predictor importance to the model. In addition, we also chose to select auto features as these produced smooth response curves. We opted to change the following settings: 1) we controlled over-fitting and clamping by setting the regularisation parameter to 1; 2) we evaluated the model and reduced bias by setting a random seed and selecting a random test percentage of 25 percent (i.e., the model was trained using 75% of the data); 3) we ensured variability by choosing to subsample the data over 10 replicate models; and 4) we allowed the model enough time for convergence by setting the number of iterations to 5000. The importance of individual bioclimatic predictors was assessed using jack-knife procedures and their individual percentage contribution to training the model. We evaluated model performance using a measure of model performance called the area under the curve (AUC) of the receiver operating characteristic, ranging from 0 to 1 (high accuracy = AUC > 0.9; moderate accuracy = 0.9 < AUC > 0.7; poor accuracy = 0.7 < AUC > 0.5; model performance worse than random = AUC < 0.5) (Peterson et al. 2011). We created binary maps of the species predicted climatic suitability using ESRI ArcMap 10.3.1 (ESRI, 2015). Climate is one of the main determinants of species growth and establishment in regions outside their native ranges (Welk et al. 2002, Robertson et al. 2004, Thuiller et al. 2006, Ficetola et al. 2007); therefore we utilised climatic data from the WORLDCLIM database (19 bioclimatic predictors – http://www.worldclim.org/) (Hijmans et al. 2005). We selected bioclimatic predictors which were closely related to the successful growth and establishment of the selected species (e.g. *Lithobates catesbeianus* thrives in wet, hot environments, therefore we selected precipitation of the warmest month as a climatic variable), and those predictors which were least correlated. We tested the multicollinearity of the data for each species using the correlation and summary statistics tool found in the SDM toolbox developed for ESRI ArcMap (Brown, 2014). The SDM toolbox was developed to facilitate the pre-processing of data for species distribution modelling, specifically using the Maxent software (Phillips et al. 2008, Brown 2014). The correlation between raster layers is measured as the dependency between all of the input layers. Correlation was measured as a ratio of the covariance between the raster layers divided by the product of their standard deviations. We set a correlation cut-off value of 0.60 (i.e., layers with a correlation of 0.60 or higher were considered as being highly correlated) (Snedecor and Cochran 1968, Brown 2014). Layers which were highly correlated were excluded from the climatic models.

Results

Fifty-nine species were on both the NEMBA prohibited species list and the watch list produced by Faulkner et al. (2014) (invertebrates – 9, plants – 32 and vertebrates – 18). Based on the Köppen-Geiger Climate Classification (Köttek et al. 2006), there are 39 cities of over a million inhabitants which have the same climate type as eThekwini (Suppl. material 1). Ten species, from the initial 59, were present in at least one of the 39 cities. After eliminating species which were only alien or invasive on islands, five species were left (*Alternanthera philoxeroides* – alligator weed, *Cenchrus echinatus* – southern sandbur, *Lithobates catesbeianus* – American bullfrog, *Solenopsis invicta* – red imported fire ant, and *Vulpes vulpes* – red fox).

We identified the pathways of introduction for each of the remaining species. At this stage, we excluded *V. vulpes* (red fox) as it is extremely unlikely to be introduced by the only pathways that have historically led to its introduction to other countries (hunting in the wild and fur farms – GISD, 2018). The pathways facilitating the introduction of *C. echinatus* were unknown (GISD, 2018). This meant that while it was possible to still build a climatic suitability model for the species, it is not possible, at this stage, to link climate suitability to introduction pathways (Box 2). *Alternanthera philoxeroides* (Box 1) and *S. invicta* (Box 4) have previously been introduced through the transport-stowaway and transport-contaminant pathways. The introduction of *L. catesbeianus* (Box 3) has been facilitated through the release and escape pathways. Three main potential points of introduction were identified for these species based on the pathways: the Durban Harbour (all four species), pet and aquarium stores (29 within the municipal boundary – *L. catesbeianus*) as well as plant nurseries and garden centres (60 within the municipal boundary – *S. invicta*). We then identified likely points of first naturalisation as sites to monitor for the presence of the three species: the Durban Harbour was identified as a site to monitor for the presence of *A. philoxeroides* (Figure B1) and *S. invicta* (Figure B4). River systems adjacent to points of introduction are also identified for surveillance efforts for *A. philoxeroides* (Figure B1), *L. catesbeianus* (Figure B3) and *S. invicta* (Figure B4) because of these species' dependency on readily available water resources for survival. We also identified the built infrastructure surrounding the Durban Harbour for monitoring for *S. invicta* (Figure B4). River systems and wetlands adjacent to pet and aquarium stores were identified for monitoring for the presence of *L. catesbeianus* (Figure B3).

Species distribution models

The climate models developed for the selected species ranged from highly accurate model performance to moderately accurate performance based on the AUC of receiver

Box 1. Pathways of introduction, preferred habitats, potential entry points, sites to monitor, and climatic suitability for *Alternanthera philoxeroides* (alligator weed).

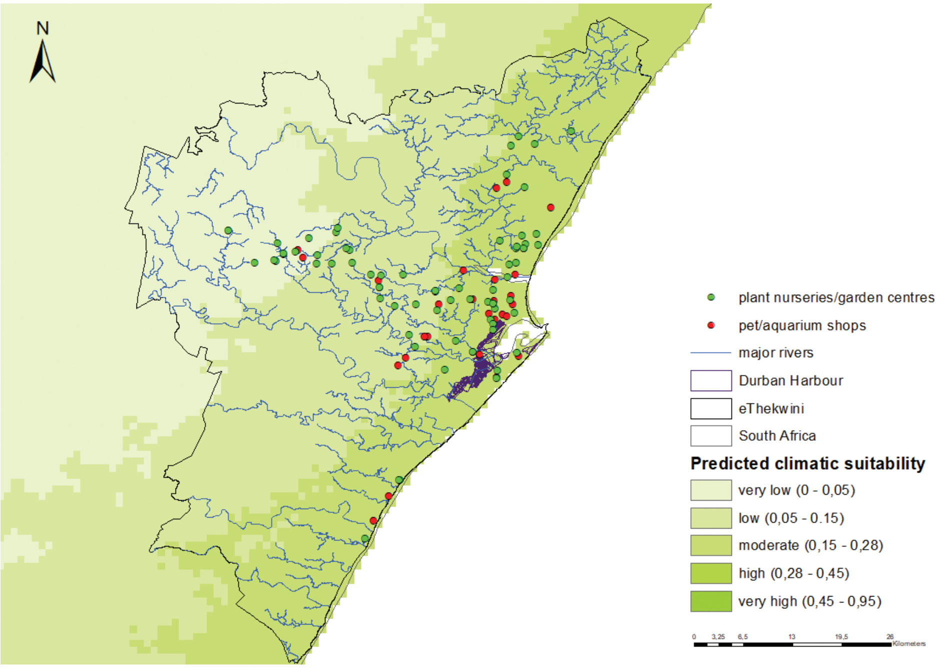

Figure B1. Predicted climatic suitability *A. philoxeroides* in Durban. The model is highly accurate in predicting climatic suitability (0.929 ± 0.007 – AUC±SD). Predicted suitability is indicated using a colour scale (darker shades indicate higher predicted suitability). Also indicated are the potential points of introduction and potential points of first naturalisation to monitor for *A. philoxeroides* in Durban.

Pathways of introduction: Ship ballast (historical), transportation of habitat material, ornamental purposes

Potential points of first introduction: The Durban harbour, plant nurseries and garden centres, pet and aquarium shops

Habitat and Land uses: *Alternanthera philoxeroides* can grow in a variety of habitats but is usually found in aquatic habitats, particularly rivers, lakes, dams, ponds, canals, flood plains and irrigation channels

Habitats present in Durban: Yes

Potential sites of first naturalisation in Durban: The Durban harbour and adjacent river systems (particularly uMhlatuzana and uMbilo river systems)

Box 2. Pathways of introduction, preferred habitats, potential entry points, sites to monitor, and climatic suitability for *Cenchrus echinatus* (southern sandbur).

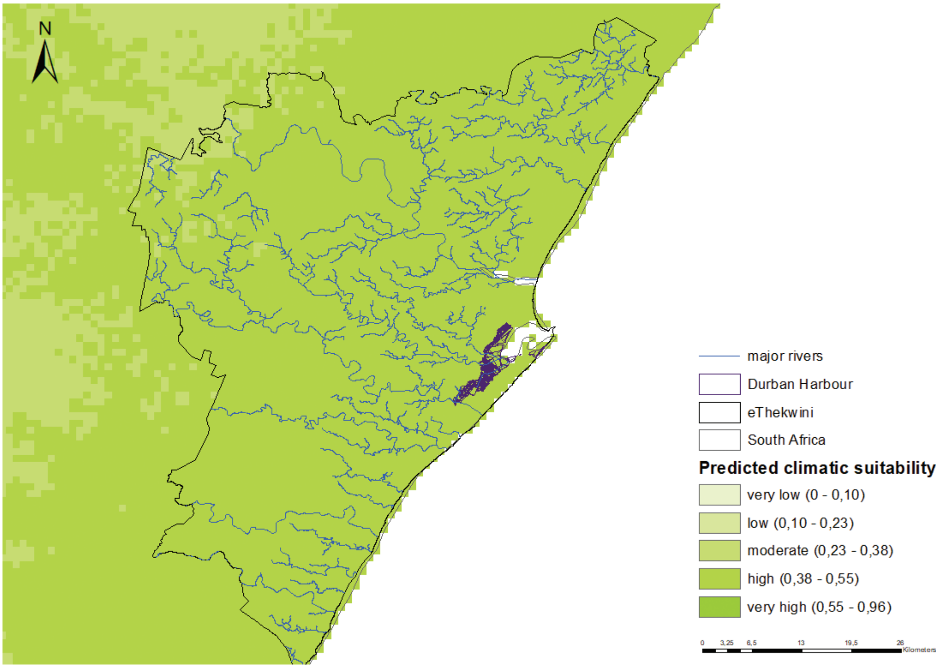

Figure B2. Predicted climatic suitability for *C. echinatus* in Durban. The model is moderately accurate in predicting climatic suitability (0.812 ± 0.008 – AUC±SD). Predicted climatic suitability is indicated using a colour scale (darker shades indicate higher predicted suitability). Even though pathways of introduction for this species could not be identified with certainty, the potential points of introduction and first naturalisation (i.e. where to monitor) for *C. echinatus* in Durban are indicated.

Pathways of introduction: Unknown

Potential points of first introduction: The Durban harbour

Habitat and Land uses: *Cenchrus echinatus* favours temperate and tropical zones. This species is usually found in open lands, cultivated fields, along roadsides and coastal environments and waste places.

Habitats present in Durban: Yes

Potential sites of first naturalisation: The Durban harbour and adjacent beach environments and sand dunes

Box 3. Pathways of introduction, preferred habitats, potential entry points, sites to monitor, and climatic suitability for *Lithobates catesbeianus*.

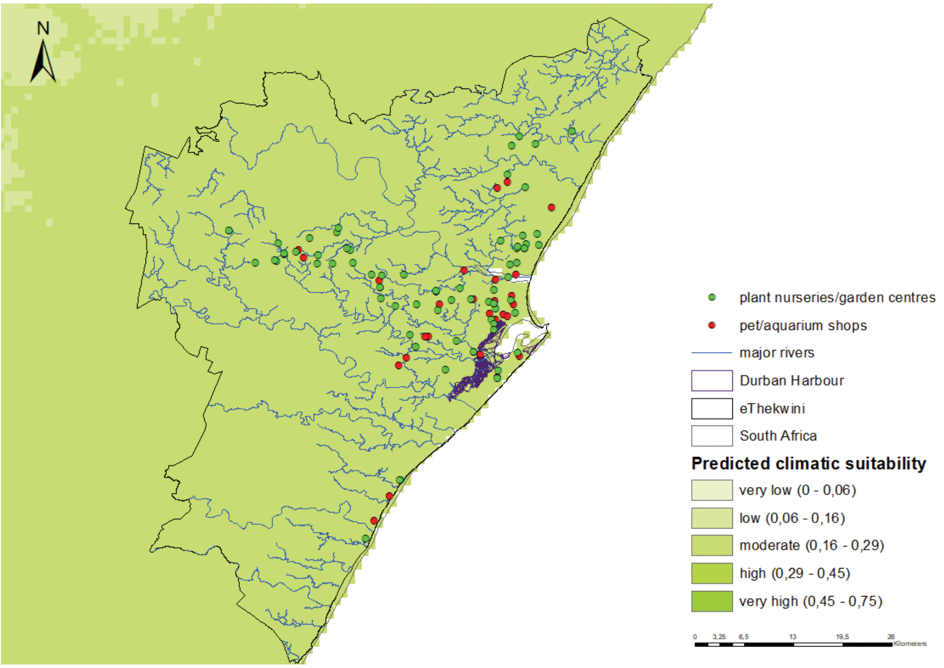

Figure B3: Predicted climatic suitability of *L. catesbeianus* in Durban. The model is moderately accurate in predicting climatic suitability (0.791 ± 0.005 – AUC±SD). Predicted suitability is indicated using a colour scale (darker shades indicate higher predicted suitability). Also indicated are the potential points of first naturalisation (i.e. priorities for monitoring) for *C.echinatus* in Durban.

Pathways of introduction: Biological control, landscape; floral and faunal improvement, release in use for nature, aquaculture (food source), ornamental purposes

Potential points of first introduction: The Durban harbour, pet and aquarium shops

Habitat and Land uses: *Lithobates catesbeianus* prefers warm, moist environments and requires permanent, shallow and still bodies of water. This frog species usually occupies ponds, swamps, streams and irrigation ditches

Habitats present in Durban: Yes

Potential sites of first naturalisation: Major river systems, especially those adjacent to potential points of introduction (pet and aquarium shops)

Box 4. pathways of introduction, preferred habitats, potential entry points, sites to monitor, and climatic suitability for *Solenopsis invicta*.

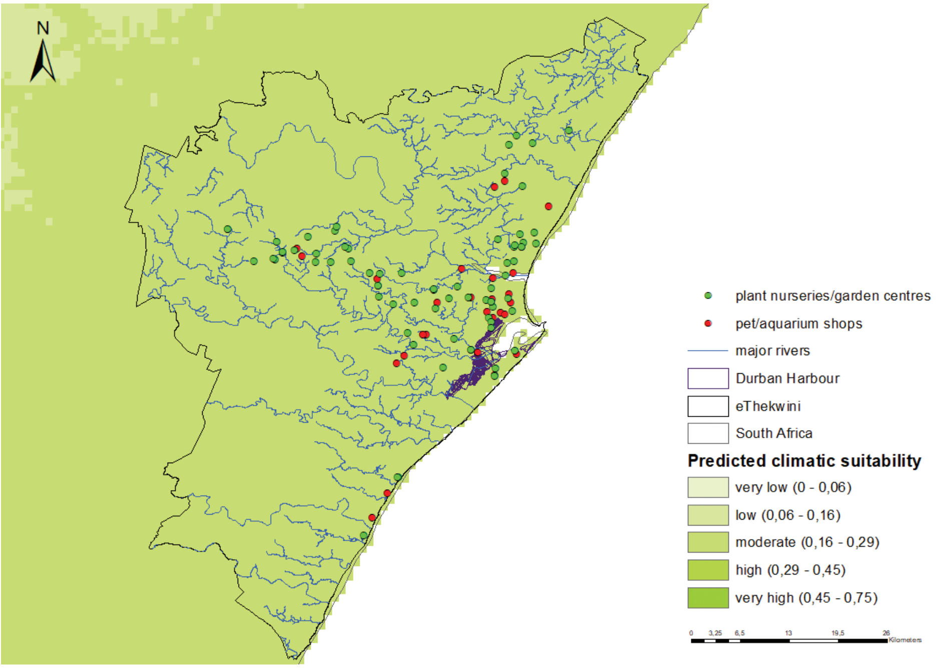

Figure B4: Predicted climatic suitability of *S. invicta* in Durban. The model is highly accurate in predicting climatic suitability (0.961 ± 0.006 – AUC±SD). Predicted suitability is indicated using a colour scale (darker shades indicated higher predicted suitability). Also indicated are the potential points of introduction and fist naturalisation to monitor for *S. invicta* in Durban.

Pathways of introduction: Contaminated nursery material, translocation of machinery and equipment, organic wood packaging

Potential points of first introduction: The Durban harbour, plant and nursery material

Habitat and Land uses: *Solenopsis invicta* can occupy a wide variety of habitats and can become dominant in altered habitats. This ant species is found in disturbed or developed forests or on trails near buildings

Habitats present in Durban: Yes

Potential sites of first naturalisation: The Durban harbour and adjacent built infrastructure, plant nurseries and garden centres and surrounding natural environments linked to major river systems operating characteristics (see Table 1 for details). However, the patterns of predicted climatic suitability varied for each of the species. The *L. catesbeianus* (Figure B3) and *C. echinatus* (Figure B2) models (moderately accurate performance) showed a uniform climatic suitability for these species across the city, with *C. echinatus* having a higher predicted climatic suitability than *L. catesbeianus*. The *A. philoxeroides* (Figure B1 – highly accurate model performance) model showed the highest predicted climate suitability along the coastline of eThekwini decreasing to the north-west of the city. The *S. invicta* (Figure B4 – highly accurate model performance) model showed a relatively low climatic suitability; however, the most important regions for *S. invicta* were the northern regions and the coastline of the city (see Table 1 for details).

Additionally, we superimposed pet and aquarium shops, nurseries and garden centres, the major river systems and the Durban Harbour data with the climatic suitability models (see Boxes 1–4). From the sixty plant nurseries and garden centres in eThekwini, eighteen were located adjacent to major rivers, while seven were located adjacent to the Durban Harbour. Climatic suitability for *C. echinatus* and *L. catesbeianus* (Boxes 2–3) was found to be uniform across the city; therefore, all points of introduction are likely to be sites of first naturalisation. The highest predicted climatic suitability for *A. philoxeroides* (Box 1) was found along the coast of eThekwini in which 34 plant nurseries and garden centres were located. We found 23 plant nurseries and garden centres located in low climate suitability regions for *S. invicta* (Box 4). We found 29 pet and aquarium shops within eThekwini, 13 of which were located near the major river systems while eight were located near the harbour. Nineteen pet and aquarium shops were located in the regions of highest predicted suitability for *A. philoxeroides*, while 17 were

Table 1. List of species for which predictive models were developed, the bioclimatic predictors used to develop each model, and the percentage contribution of each predictor to the model.

Species	Bioclimatic Predictors selected (% contribution to model)	Model Performance (AUC ± Standard Deviation)
Alternanthera philoxeroides	Mean diurnal range (10), Mean temperature of the warmest month (17), Precipitation seasonality (21), Precipitation of the warmest quarter (9), Precipitation of the coldest quarter (54)	High accuracy (0.929 ± 0.007)
Cenchrus echinatus	Mean temperature of the warmest quarter (25), Precipitation of seasonality (34), Precipitation of the wettest quarter (44), Precipitation of the driest quarter (7)	Moderate accuracy (0.812 ± 0.008)
Lithobates catesbeianus	Mean diurnal range (4), Temperature seasonality (44), Maximum temperature of the warmest month (21), Precipitation of the warmest quarter (3), Precipitation of the coldest quarter (38)	Moderate accuracy (0.791 ± 0.005)
Solenopsis invicta	Mean diurnal range (13), Maximum temperature of the warmest month (28), Precipitation of the wettest month (20), Precipitation of the driest month (45), Precipitation seasonality (4)	High accuracy (0.961 ± 0.006)

located in the highest predicted suitability for *S. invicta*. One pet and aquarium shop was located within the built infrastructure adjacent to the Durban Harbour; hence this was highlighted as an important potential point of introduction for *A. philoxeroides*, *L. catesbeianus* and *S. invicta*.

Discussion

While watch lists and prohibited lists are beneficial in highlighting species to monitor, the lists often consist of numerous species, across a variety of taxa (e.g. the NEMBA prohibited species list – 553 targeted species; Faulkner et al. 2014 – 400 watch list species). The selection criteria used in this study (Figure 1) allow for these lists to be narrowed down in the context of a specific urban setting, to provide priority targets for incursion response. We recommend that three of the species identified (*Alternanthera philoxeroides*, *Lithobates catesbeianus* and *Solenopsis invicta*) be targeted for contingency planning in eThekwini, e.g. through the production of awareness material to improve passive surveillance, consideration of active surveillance through a monitoring scheme, and the development of incursion response plans so that if they are detected, there is no delay before action is taken (Wilson et al. 2017). Consideration should also be given to planning for the fourth species, *Cenchrus echinatus*, although the priority will be to first identify if and where it is likely to be introduced.

The Convention on Biological Diversity (CBD) Aichi Target 9 requires that pathways of introduction be identified and prioritised for management efforts (UNEP, 2011). In this study, we identified likely sites of first naturalisation as priorities for incursion response efforts. We identified three important potential introduction points: the Durban Harbour; pet and aquarium stores; and nursery and garden centres. Each of the species used in this study were linked to one of these potential introduction points. The potential sites of first naturalisation identified in this study were all found to be in close proximity to the Durban harbour and the major river systems in the city, indicating that these sites are important for monitoring efforts.

Identifying the pathways facilitating the introduction of alien species is important for preventing alien species introductions. However, not all pathways of introduction are operational in all cities. By identifying the pathways which facilitate alien species introductions, priorities can be assigned to species with the potential of being introduced to the particular region of interest. In this study we were able to eliminate the species *Vulpes vulpes* (red fox) because the pathways facilitating its introduction (hunting in the wild and fur farms) are not operational in eThekwini. By contrast, the pathways which facilitate the introduction of *C. echinatus* are unknown. Therefore, determining if, how, and where the species is likely to be introduced to the city should be a key area for future applied research.

The Durban Harbour was identified as an important potential introduction point as well as a site to monitor for the introduction of *A. philoxeroides* and *S. invicta*. The pathways facilitating the introduction of these species are linked to the harbour. *Alternanthera philoxeroides* is primarily introduced through ship ballast and as a stowaway on ship cargo (Burgin et al. 2010), while *S. invicta* is introduced on organic wood packaging. These species can thrive in highly transformed habitats; therefore we also recommend the adjacent infrastructure to the harbour as sites for monitoring efforts. *S. invicta* is known to have negative ecological, economic and social impacts (Tang et al. 2013). Ecologically, this species is known to reduce native invertebrate and vertebrate communities through predation (Allen et al. 2004, McGlynn 1999, Holway et al. 2002). Furthermore, this species dominates altered habitats such as those present in cities, where *S. invicta* has an affinity to electrical equipment (Morrison et al. 2004). This ant is considered to be one of the most destructive invasive ant species (Lowe et al. 2000, Ascune et al. 2011). *S. invicta* also has negative social impacts and poses a threat to humans as the venom from *S. invicta* stings can cause severe allergic reactions (Solley et al. 2002). Box 4 shows that predicted climatic suitability for *S. invicta* coincides with land use in the city; this is potentially problematic for the human population. Therefore, we recommend that this species should be a priority target for strategic prevention efforts.

The river systems adjacent to potential point of introduction in the municipality were also identified as important sites to monitor. *Alternanthera philoxeroides* (Julien et al. 1995) and *Lithobates catesbeianus* (da Silva and Filho 2009) are found in aquatic habitats such as rivers, along flood plains, in lakes and dams. *Alternanthera philoxeroides* is primarily an aquatic plant but can invade terrestrial environments such as agricultural areas (Burgin et al. 2010). *Alternanthera philoxeroides* can reproduce vegetatively to form new infestations from broken plant material and often forms fragile mats covering water bodies. *Lithobates catesbeianus* is introduced primarily through intentional introductions for faunal improvement to landscapes, ornamental purposes and through aquaculture as a food source (Measey et al. 2017). *Lithobates catesbeianus* has high fecundity and environmental plasticity and is known to grow relatively large in size, ensuring their survival in a variety of habitats including disturbed environments (da Silva and Filho 2009, Akmentins and Cardozo 2010). Furthermore, bullfrogs are potential vectors of diseases to native amphibians (Ficetola et al. 2007, Eskew et al. 2015). Box 1 (*A. philoxeroides*) and Box 3 (*L. catesbeianus*) both show potential points of introduction in close proximity to the major river systems in the municipality. Both of these species are considered to be prolific invaders with potentially devastating impacts (*A. philoxeroides* – Burgin and Norris 2008, Chen et al. 2013, *L. catesbeianus* – Lowe et al. 2000). Both *A. philoxeroides* (Burgin and Norris 2008, Burgin et al. 2010, Basset et al. 2010, Clements et al. 2011) and *L. catesbeianus* (Ficetola et al. 2007, da Silva and Filho 2009, da Silva et al. 2009) are capable of spread via natural dispersal once introduced and will be at best difficult to manage (Padayachee et al. 2017), especially because the likelihood of these species establishing throughout the city is high (Boxes 1, 2). We recommend both of these species as targets for strategic prevention efforts in eThekwini.

Invasions are, of course, often unpredictable and context dependent. Therefore the prioritisation here should only be one small part of an overall biosecurity strategy (Wilson et al. 2017). The most effective methods for detection (e.g. traps or visual inspections) and the mix between passive and active surveillance (Hester and Cacho 2017) will depend on the biology of the organism. Similarly, it is important to understand the context of the invasion, going beyond whether pathways still operate to consider factors that might limit invasions (e.g. is there a strong mechanistic reason , such as biotic resistance, for expecting that the uniquely insular invasions discounted here will not become invasive in eThekwini?). It will be vitally important to continue general surveillance efforts and create and maintain capacity to respond to surprises. However, by identifying species that are known to be problematic elsewhere in the world, that are likely to establish in eThekwini, and that are likely to be introduced, at least part of the detection and response efforts can be prioritised. It also helps eThekwini meet its legal requirements to address the threat posed by future biological invasions.

Even though this study focuses on eThekwini, the procedures used here represent a practical method to assign priorities for preventing the introduction of alien species. The methodology used in this study has merit for assigning priorities to a variety of taxa, such as this study (invertebrates, plants and vertebrates), or single taxa studies. Online databases such as CABI ISC, GBIF, GISD and GRIIS make alien species information required for utilising this methodology readily accessible. The accessibility of information and adaptability of the methodology used in this study makes the protocol feasible. However, there are many ways in which the protocol can be improved. For example, occurrence data sourced from online databases are often plagued with inconsistencies (e.g. validity of location points and taxonomy). The use of expert opinion in determining the validity of these data is a potentially beneficial improvement to this prioritisation tool. The procedures used in this study can further be improved quantitatively through additional analyses which will assess how pathways of introduction contribute to invasiveness (e.g. frequency analysis tests) of the target species as well as the contribution of potential introduction points to invasiveness (e.g. landscape level analysis) of target species. The advantage of the technique presented here is that it focuses on likely known threats and ensures that appropriate measures are put in place to deal with them.

Conclusion

Prioritisation is a fundamental component of effective strategic prevention strategies targeting the introduction of alien species to new regions (Reaser et al. 2008, Essl et al. 2011, McGeoch et al. 2016, Padayachee et al. 2017, Pergl et al. 2017). The selection criteria used in this study provide decision makers with an easy way to identify where to focus resources to target incursions that have a high likelihood of occurring and resulting in substantial negative impacts. Implementing prioritisation schemes that consider all three aspects (species, pathways, and sites) (Wilson et al. 2017) allows decision makers to target monitoring efforts where the risk of particular invasions is highest. Additionally, integrating prioritisation schemes, such as in this study, allows decision makers to focus resources on species which poses a greater risk of invasion and impact.

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Advancing research on alien species and biological invasions

A citation-based map of concepts in invasion biology

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Abstract

Invasion biology has been quickly expanding in the last decades so that it is now metaphorically flooded with publications, concepts, and hypotheses. Among experts, there is no clear consensus about the relationships between invasion concepts, and almost no one seems to have a good overview of the literature anymore. Similar observations can be made for other research fields. Science needs new navigation tools so that researchers within and outside of a research field as well as science journalists, students, teachers, practitioners, policy-makers, and others interested in the field can more easily understand its key ideas. Such navigation tools could, for example, be maps of the major concepts and hypotheses of a research field. Applying a bibliometric method, we created such maps for invasion biology. We analysed research papers of the last two decades citing at least two of 35 common invasion hypotheses. Co-citation analysis yields four distinct clusters of hypotheses. These clusters can describe the main directions in invasion biology and explain basic driving forces behind biological invasions. The method we outline here for invasion biology can be easily applied for other research fields.

Keywords

bibliometric methods, biological invasions, concepts, invasion biology, invasion science, map, navigation tools, network of invasion hypotheses

Introduction

When you are visiting a city, you can usually find some important places by yourself, for example the central station, a supermarket, and maybe even a few touristic highlights. A better way, however, would be that a friend draws you a map with the places in the city you are interested in. Then you would also find the small French café, the little arthouse cinema, and the restaurant serving delicious oriental food. But this map will be limited by your friend's knowledge of her district. What if you want to visit another part of the city? You will find yourself in the same position as before. Therefore, an even better way is to ask several people who live in different areas of the city. In this way, you can get a detailed picture of the whole city and, if you are lucky, even find the best brewed coffee in the city.

The same is true when you start in a new research field. Enders et al. (2018) showed that the field of invasion biology can be seen as such a big city in which many of its inhabitants, i.e. invasion biologists, have no clear picture of the whole city; their knowledge seems to be limited to their immediate field of interest within invasion biology. What is the solution for a problem like this? Suppose you have no good tourist guide at hand, then you need to observe where other tourists go to and follow them. For a research field, this would be an analysis of citations made by specialists.

Authors of a scholarly paper cite publications and other sources they assume to be relevant for the topic of their paper. Thus, scholarly papers form a huge network, a view already propagated by one of the fathers of bibliometrics (de Solla Price 1965). The identification of topics in bibliographies is an old problem in bibliometrics. Starting with co-citation analysis (Marshakova 1973; Small 1973; Small and Sweeny 1985), important recent developments include hybrid approaches that combine citation-based and term-based techniques (Glenisson et al. 2005; Glänzel and Thijs 2017), and term-based probabilistic methods (topic modelling, Yau et al. 2014). The 21st century brought the advance of many methods for clustering in networks (Fortunato 2010; Xie et al. 2013; Amelio and Pizzuti 2014). Some of these methods were also applied to citation networks (Gläser et al. 2017; Velden et al. 2017), and topic identification is often accompanied by visualization of the topic landscape (Börner 2015).

For this publication, we analysed co-citations of invasion hypotheses in research papers of the last two decades. Co-citation analysis was independently introduced by Irina Marshakova (1973) and Henry Small (1973) (see also Havemann 2016). Because there are no strict rules for citing, they had to solve the problem of noise in co-citation data. Irina Marshakova compared the observed absolute co-citation numbers with expected numbers in a null model of independent random citing and only accepted co-citation links between cited sources that are more frequently co-cited than in 95% of random trials in the null model. In other words, she assumed binomial distributions of co-citation numbers and chose a significance level of 95%. Henry Small, on the other hand, reduced noise by using thresholds of relative co-citation measures (Jaccard and Salton index). Also, other relative measures of co-citation strengths were used (Gmür 2003; Egghe and Leydesdorff 2009; Boyack and Klavans 2010). In a recent study, Trujillo and Long (2018) used absolute co-citation numbers as a similarity measure and created a sequence of nested co-citation networks by setting different thresholds for this measure. In invasion biology or related research fields, however, no citation-based map of major concepts and hypotheses does, to our knowledge, currently exist.

Invasion biology is a discipline that grew very slowly at first. In the 19th century, early concepts on non-native species were mentioned (Cadotte 2006), for example in Darwin's (1859) book "On the origin of species by means of natural selection". Further concepts were introduced by the Swiss botanist Albert Thellung (Kowarik and Pyšek 2012), Elton (1958) and others until the 1950s; however, there was still too little work on the topic to recognize a distinct research field. Possibly due to a growing consciousness for ecosystems in a changing world (Meadows et al. 1972) and in human responsibilities (Jonas 1979), interest in invasion biology strongly increased since the late 20th century (Richardson and Pyšek 2008). It has also influenced other research fields; for example, concepts and hypotheses of invasion biology are used in restoration ecology, landscape ecology, urban ecology, or risk assessments of genetically modified organisms (Jeschke et al. 2013; Lowry et al. 2013).

Our study aims were twofold. First, we wanted to find a suitable map of the field of invasion biology based on co-citation analysis. Second, we aimed to compare this map to those created with two other approaches: a map based on an assessment of the characteristics ("traits") of hypotheses (Enders and Jeschke 2018), and one based on an online survey (Enders et al. 2018).

Methods

We defined 35 common concepts and hypotheses in invasion biology and their representing key publications (Table 1). This list is based on Enders and Jeschke (2018) and Enders et al. (2018), which are in turn based on Catford et al. (2009). For clarity, we only give one key publication per hypothesis. One paper is the key publication for four hypotheses (EI, ERD, IS, NAS), and another paper for two hypotheses (SG, BID) (Table 1). Thus, Table 1 includes 31 key publications.

A first hint about relationships between our key publications can be obtained from their direct citation links, but this approach is limited by the small sample size of publications. As there is some randomness in the act of citation, a larger sample size is useful. Using bibliographic coupling relations between key papers, i.e., analysing to which degree their reference lists overlap, has the same drawback.

An alternative approach, which we applied here, is co-citation analysis, where joint citations of key papers are analysed, using all publications of the field. This approach can thus draw from a much larger dataset.

We downloaded all 10,430 records citing any of our key publications from the Web of Science (WoS, as licensed for Freie Universität Berlin, March 2017). Variants of referencing key papers were identified semi-automatically with the help of an R-script provided by Felix Mattes. For example, missing or wrong author initials or wrong page numbers were corrected in this way. Then we determined the yearly citation and co-citation numbers of all key publications. We expect higher numbers of key papers cited in review papers which diminishes the weight of each co-citation. Therefore, we excluded reviews from the analysis.

Key invasion papers are also cited outside of invasion biology. We excluded such outside-of-the-field papers from co-citation analysis, as invasion hypotheses are primarily applied in invasion biology and we expect that peculiarities of their relationships are discussed within the field, whereas joint citations by publications outside of the field are less reliable for assessing such relationships. We defined papers belonging to the field as those that are returned by the term search proposed by Vaz et al. (2017):

"Ecological invasion" or "Biological invasion*" or "Invasion biology" or "Invasion ecology" or "Invasive species" or "Alien species" or "Introduced species" or "Non-native species" or "Nonnative species" or "Nonindigenous species" or "Non-indigenous species" or "Allochthonous species" or "Exotic species".*

Using this term search on 28.08.2017 in the WoS returned 30,731 records. After excluding 1,769 review papers, 28,962 papers remained in the sample. These are mainly primary research communications (28,295) and have mainly been published after 1990 (28,841; i.e. 99.6%). Figure 1 displays the time distribution of the sample of these 28,841 invasion biology papers in the WoS. In the 1990s, the number of papers in the field has remained small. We therefore restricted our analysis to the time period 1999–2017. Thus, we ended up with a sample of 1,518 invasion biology papers that cite at least two of our key publications listed in Table 1. The sample includes 1501 research articles, mainly in journals but also 39 in conference proceedings and five in books. In addition, we have eight letters and nine editorials. The time distribution of the sample is displayed in Figure 2.

Salton's cosine

In the n-dimensional vector space with one dimension per citing paper, each cited source i can be represented by a vector v_{ik} ($k = 1, ..., n$) with $v_{ik} = 1$ if paper k cites source i and $v_{ik} = 0$ otherwise. The Salton index $S(i, j)$ of two sources is a similarity measure defined as the cosine of the angle between the two source vectors (Hamers et al. 1989). Translated into the language of set theory, it can be calculated as:

$$S(i,j) = \frac{\left|c_i \cap c_j\right|}{\sqrt{\left|c_i\right| \cdot \left|c_j\right|}}, \tag{1}$$

where c_i is the set of papers citing source publication i. Salton's cosine gives values in the interval [0, 1]. Co-citations are usually determined within reference lists of citing publications c_i published during a given year. Due to heavily fluctuating citation num-

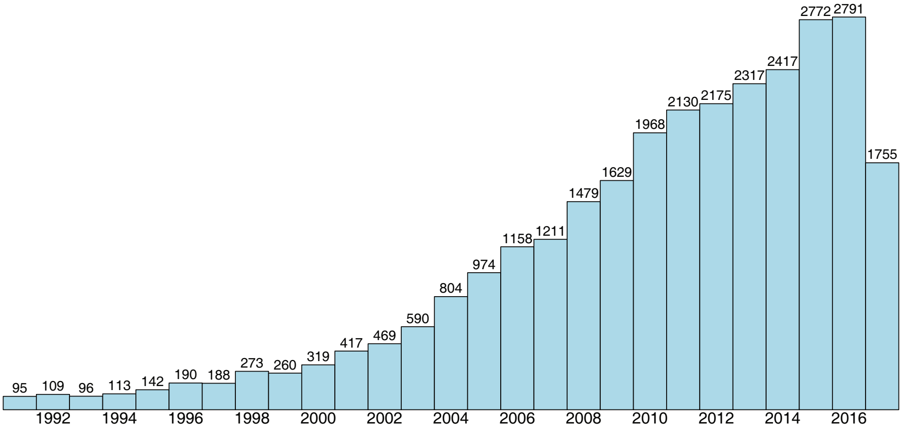

Figure 1. Numbers of publications in invasion biology, using the same search term as Vaz et al. (2017) in the Web of Science. The number of publications in 2017 is relatively low because the search was performed within this year, on 28 August 2017.

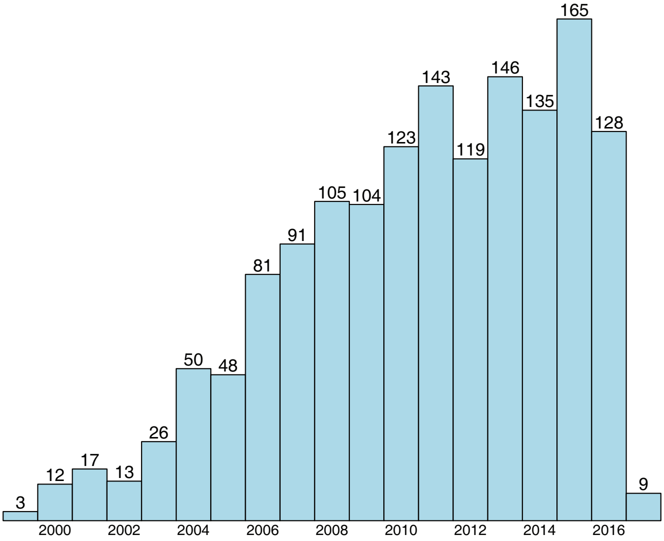

Figure 2. Number of publications per year that cite at least two of the key papers given in Table 1. This sample of 1518 publications was analysed in detail here; it is a subset of the publications shown in Figure 1.

bers, we combined several years to get broader citation windows. Due to this change, a challenge was that two key papers i and j published within the citation window in different years $y_i < y_j$ have different chances to be cited: older papers have more opportunities to be cited than younger papers. We made their chances to be cited as equal as possible by reducing the set c_i to citing papers published from year y_j on.

Table 1. List of 35 common invasion hypotheses and how we defined them (cf. Catford et al. 2009; Enders and Jeschke 2018; Enders et al. 2018).

Hypothesis		Description	Key reference
ADP	Adaptation	The invasion success of non-native species depends on the adaptation to the conditions in the exotic range before and/or after the introduction. Non-native species that are related to native species are more successful in this adaptation.	Duncan and Williams (2002)
BA	Biotic acceptance aka "the rich get richer"	Ecosystems tend to accommodate the establishment and coexistence of non-native species despite the presence and abundance of native species.	Stohlgren et al. (2006)
BID	Biotic indirect effects	Non-native species benefit from different indirect effects triggered by native species.	Callaway et al. (2004)
BR	Biotic resistance aka diversity-invasibility hypothesis	An ecosystem with high biodiversity is more resistant against non-native species than an ecosystem with lower biodiversity.	Levine and D'Antonio (1999)
DEM	Dynamic equilibrium model	The establishment of a non-native species depends on natural fluctuations of the ecosystem, which influences the competition of local species.	Huston (1979)
DN	Darwin's naturalization	The invasion success of non-native species is higher in areas that are poor in closely related species than in areas that are rich in closely related species.	Daehler (2001)
DS	Disturbance	The invasion success of non-native species is higher in highly disturbed than in relatively undisturbed ecosystems.	Hobbs and Huenneke (1992)
EE	Enemy of my enemy aka accumulation-of-local-pathogens hypothesis	Introduced enemies of a non-native species are less harmful to the non-native as compared to the native species.	Eppinga et al. (2006)
EI	Enemy inversion	Introduced enemies of non-native species are less harmful for them in the exotic than the native range, due to altered biotic and abiotic conditions.	Colautti et al. (2004)
EICA	Evolution of increased competitive ability	After having been released from natural enemies, non-native species will allocate more energy in growth and/or reproduction (this re-allocation is due to genetic changes), which makes them more competitive.	Blossey and Nötzold (1995)
EN	Empty niche	The invasion success of non-native species increases with the availability of empty niches in the exotic range.	MacArthur (1970)
ER	Enemy release	The absence of enemies in the exotic range is a cause of invasion success.	Keane and Crawley (2002)
ERD	Enemy reduction	The partial release of enemies in the exotic range is a cause of invasion success.	Colautti et al. (2004)
EVH	Environmental heterogeneity	The invasion success of non-native species is high if the exotic range has a highly heterogeneous environment.	Melbourne et al. (2007)
GC	Global competition	A large number of different non-native species is more successful than a small number.	Colautti et al. (2006)
HC	Human commensalism	Species that are living in close proximity to humans are more successful in invading new areas than other species.	Jeschke and Strayer (2006)
HF	Habitat filtering	The invasion success of non-native species in the new area is high if they are pre-adapted to this area.	Weiher and Keddy (1995)
IM	Invasional meltdown	The presence of non-native species in an ecosystem facilitates invasion by additional species, increasing their likelihood of survival or ecological impact.	Simberloff and Von Holle (1999)
IRA	Increased resource availability	The invasion success of non-native species increases with the availability of resources.	Sher and Hyatt (1999)
IS	Increased susceptibility	If a non-native species has a lower genetic diversity than the native species, there will be a low probability that the non-native species establishes itself.	Colautti et al. (2004)
ISH	Island susceptibility hypothesis	Non-native species are more likely to become established and have major ecological impacts on islands than on continents.	Jeschke (2008)
IW	Ideal weed	The invasion success of a non-native species depends on its specific traits (e.g. life-history traits).	Rejmánek and Richardson (1996)

Hypothesis		Description	Key reference
LS	Limiting similarity	The invasion success of non-native species is high if they strongly differ from native species, and it is low if they are similar to native species.	MacArthur and Levins (1967)
MM	Missed mutualisms	In their exotic range, non-native species suffer from missing mutualists.	Mitchell et al. (2006)
NAS	New associations	New relationships between non-native and native species can positively or negatively influence the establishment of the non-native species.	Colautti et al. (2006)
NW	Novel weapons	In the exotic range, non-native species can have a competitive advantage against native species because they possess a novel weapon, i.e. a trait that is new to the resident community of native species and therefore affects them negatively.	Callaway and Ridenour (2004)
OW	Opportunity windows	The invasion success of non-native species increases with the availability of empty niches in the exotic range, and the availability of these niches fluctuates spatio-temporally.	Johnstone (1986)
PH	Plasticity hypothesis	Invasive species are more phenotypically plastic than non-invasive or native ones.	Richards et al. (2006)
PP	Propagule pressure	A high propagule pressure (a composite measure consisting of the number of individuals introduced per introduction event and the frequency of introduction events) is a cause of invasion success.	Lockwood et al. (2005)
RER	Resource-enemy release	The non-native species is released from its natural enemies and can spend more energy in its reproduction, and invasion success increases with the availability of resources.	Blumenthal (2006)
RI	Reckless invader aka "boom-bust"	A non-native species that is highly successful shortly after its introduction can get reduced in its population or even extinct over time due to different reasons (such as competition with other introduced species or adaptation by native species).	Simberloff and Gibbons (2004)
SDH	Shifting defence hypothesis	After having been released from natural specialist enemies, non-native species will allocate more energy in cheap (energy-inexpensive) defenses against generalist enemies and less energy in expensive defenses against specialist enemies (this re-allocation is due to genetic changes); the energy gained in this way will be invested in growth and/or reproduction, which makes the non-native species more competitive.	Doorduin andVrieling (2011)
SG	Specialist-generalist	Non-native species are more successful in a new region if the local predators are specialists and local mutualists are generalists.	Callaway et al. (2004)
SP	Sampling	A large number of different non-native species is more likely to become invasive than a small number due to interspecific competition. Also, the species identity of the locals is more important than the richness in terms of the invasion of an area.	Crawley et al. (1999)
TEN	Tens rule	Approximately 10% of species successfully take consecutive steps of the invasion process.	Williamson and Brown (1986)

Communities in networks

Clusters of highly cited sources containing often co-cited sources are assumed to represent knowledge bases of current research fronts (Small and Sweeny 1985). Such clusters are particularly useful for constructing conceptual maps that should serve as navigation tools for research fields, as they group similar concepts and hypotheses in one cluster. Especially in the last two decades, several clustering methods have been developed in network science (see Fortunato (2010) for a review). Clusters (also called modules or communities) of nodes in networks should have many internal links and comparatively few external links. In the case of weighted networks, not the number of external and internal links is compared but the sum of their weights. Identifying clusters in a network is a way of investigating its inner structure.

For the case of disjoint communities, Newman and Girvan (2004) introduced "modularity" as an evaluation function of a graph partition. It compares the actual number of internal edges of each community with the number expected in a null model without community structure. In the usual null model, each vertex is expected to have the same degree as in the original graph.

We compared the results of different algorithms for community detection from the packages SNA (Handcock et al. 2003) and igraph (Csardi and Nepusz 2006) in R (R Development Core Team 2008), which can be categorized into several types. (1) The Girvan and Newman (2002) algorithm is an example of divisive clustering (igraph function cluster_edge_betweenness). It recursively detects links with high edge betweenness and removes them from the network. The clustering dendrogram is cut at the partition with maximum modularity. (2) Clauset et al. (2004) proposed to set each node as a cluster and then merge those two subgraphs that give the highest gain in modularity; this is repeated until there is no gain in modularity anymore (igraph function cluster_fast_greedy). Again, the clustering dendrogram is cut at the partition with maximum modularity. (3) Quite similar is the approach introduced by Brandes et al. (2008) (igraph function cluster_optimal). It maximizes modularity applying an optimization algorithm from integer linear programming. (4) We also applied the Louvain algorithm designed by Blondel et al. (2008) that very quickly maximizes partition modularity (igraph function cluster_louvain), (5) the "walk trap" algorithm suggested by Pons and Latapy (2005) that assumes a random walker gets trapped in communities and calculates these "traps" (igraph function cluster_walktrap), and (6) a divisive spectral algorithm suggested by Newmann (2006) which also maximizes modularity (igraph function cluster_leading_eigen).

Beside global evaluation functions like modularity, there are also functions that evaluate cohesion and separation of each community. A community C is well separated from the rest of the network if the escape probability of a random walker is small (Fortunato 2010). It is given by the ratio of the sum of external degrees of a community's nodes to the sum of their total degrees:

$$P_{esc}(C) = \frac{k_{out}(C)}{k(C)}. \tag{2}$$

The weak definition of a community after Radicchi et al. (2004) is fulfilled when the total internal degree is greater than the total external degree. The requirements are fulfilled if $P_{esc} < 0.5$. The strong community definition requires that every node has a stronger internal than external connection.

Results

We analysed different time periods (time steps of 1–5 years), but the results varied too much to get a clear picture. This means that the edges between the nodes varied from period to period. Obviously, in different years relationships between different concepts and

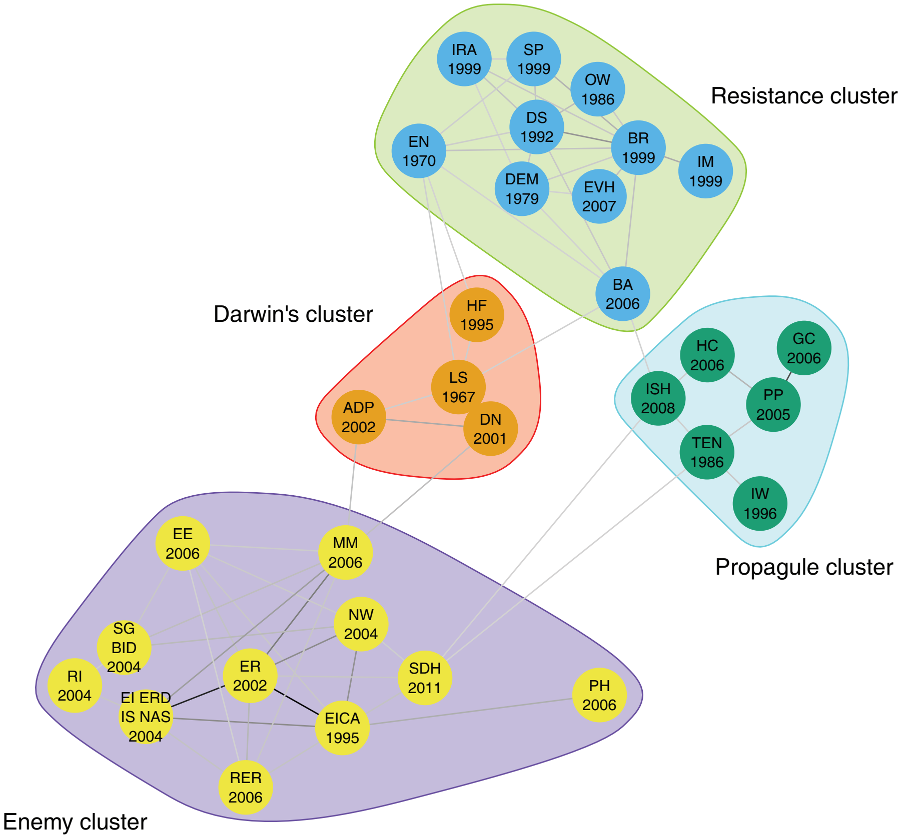

Figure 3. Partition of co-citation network *M* with maximum modularity q = 0.520. Links are weighted with significant co-citation numbers of hypothesis papers (significance level 95%, cf. text). For acronyms of hypotheses see Table 1.

hypotheses were discussed in the literature. We decided to accumulate the data from 1999 to 2017 to get a clearer, cumulative picture of relationships between invasion hypotheses.

We constructed two co-citation networks of our 31 key papers. Network *M* is based on Marshakova (1973) where accepted links are weighted by co-citation numbers (Fig. 3). Following Small and Sweeny (1985) in network *S*, we weighted all links with Salton's cosine and omitted links with a cosine below a threshold of 0.1 (Fig. 4). This threshold was chosen to receive a clearer picture of the graph and to have no unconnected nodes.

We compared the results obtained with different clustering algorithms (Table 2). In both networks, maximum modularity was achieved by a partition with four clusters. The partitions in *M* and *S* differ only in the membership of the plasticity hypothesis (PH), which switches between two clusters. We named the four clusters obtained in both networks by the most prominent principle of their hypotheses (Figs 3, 4; Table 3): *Darwin's cluster*, *resistance cluster*, *propagule cluster* and *enemy cluster*.

Table 2. Partitions of co-citation networks *M* and *S* obtained by different algorithms maximizing modularity.

Algorithm	Number of clusters		Modularity	
	M-network	*S*-network	*M*-network	*S*-network
Cluster_optimal	4	4	0.520	0.463
Fast_greedy	4	4	0.520	0.463
Louvain	4	4	0.520	0.463
Leading_eigen	6	4	0.502	0.441
Edge_betweenness	3	5	0.464	0.428
Walktrap	4	5	0.520	0.430

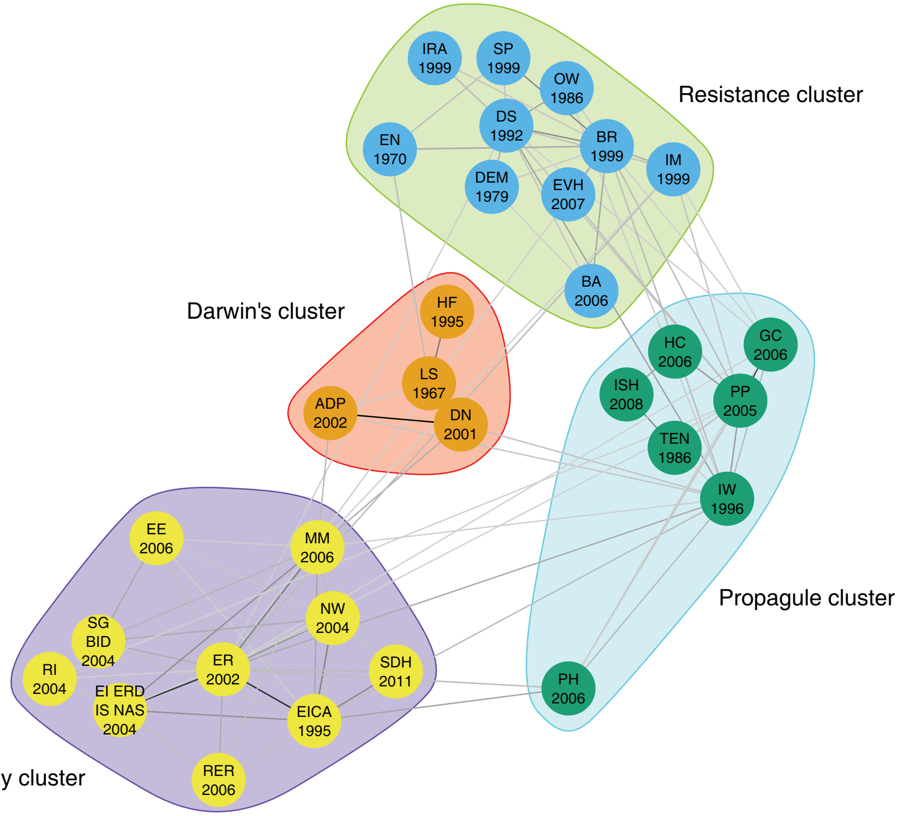

Figure 4. Partition of co-citation network *S* with maximum modularity q = 0.463. Links are weighted with Salton's cosine of co-citation numbers of hypothesis papers above a minimum threshold of 0.1 (cf. text). For acronyms of hypotheses see Table 1.

The plasticity hypothesis (PH) switches between the enemy and the propagule cluster. In the *M*-network, PH has no links to the propagule cluster because the numbers of co-citations with papers of the cluster are not significant on the 95%-level.

The best partition of network *M* has modularity 0.520 (see Fig. 3) and its clusters are communities in the weak *and* strong sense. The best partition of network *S* (Fig. 4) has modularity 0.463 and its clusters are communities in the weak sense, two of them also in the strong sense (*Darwin's* and *enemy* cluster). Since the key papers for IW (ideal weed)

Table 3. Partitions of co-citation networks *M* and *S* with maximum modularity. The key papers (see Table 1 for details) are ranked in their clusters by internal strength. Also, escape probability P_{esc} is displayed for each cluster (cf. Figs 3, 4).

Network *M*		**Network *S***	
Hypothesis-paper	**Internal strength**	**Hypothesis-paper**	**Internal strength**
Darwin´s cluster			
$P_{esc}(C) = 0.27$		$P_{esc}(C) = 0.24$	
Adaptation (ADP 2002)	54	Darwin‘s naturalization (DN 2001)	0.77
Darwin‘s naturalization (DN 2001)	53	Adaptation (ADP 2002)	0.76
Limiting similarity (LS 1967)	19	Limiting similarity (LS 1967)	0.57
Habitat filtering (HF 1995)	6	Habitat filtering (HF 1995)	0.32
Niche cluster			
$P_{esc}(C) = 0.02$		$P_{esc}(C) = 0.25$	
Biotic resistance (BR 1999)	243	Biotic resistance (BR 1999)	1.75
Disturbance (DS 1992)	163	Disturbance (DS 1992)	1.28
Sampling (SP 1999)	63	Sampling (SP 1999)	0.57
Invasional meltdown (IM 1999)	44	Biotic acceptance (BA 2006)	0.45
Increased resource availability (IRA 1999)	38	Dynamic equilibrium model (DEM 1979)	0.41
Opportunity windows (OW 1986)	37	Empty niche (EN 1970)	0.35
Biotic acceptance (BA 2006)	36	Invasional meltdown (IM 1999)	0.34
Empty niche (EN 1970)	33	Opportunity windows (OW 1986)	0.34
Dynamic equilibrium model (DEM 1979)	31	Increased resource availability (IRA 1999)	0.29
Environmental heterogeneity (EVH 2007)	24	Environmental heterogeneity (EVH 2007)	0.20
Propagule cluster			
$P_{esc}(C) = 0.01$		$P_{esc}(C) = 0.38$	
Propagule pressure (PP 2005)	186	Propagule pressure (PP 2005)	1.28
Global competition (GC 2006)	141	Global competition (GC 2006)	0.78
Human commensalism (HC 2006)	38	Ideal weed (IW 1996)	0.66
Tens rule (TEN 1986)	28	Tens rule (TEN 1986)	0.54
Island susceptibility hypothesis (ISH 2008)	11	Island susceptibility hypothesis (ISH 2008)	0.50
Ideal weed (IW1996)	10	Human commensalism (HC 2006)	0.46
		Plasticity hypothesis (PH 2006)	0.40
Enemy cluster			
$P_{esc}(C) = 0.02$		$P_{esc}(C) = 0.14$	
Enemy release (ER 2002)	652	Enemy release (ER 2002)	2.41
Evolution of increased competitive ability (EICA 1995)	465	Evolution of increased competitive ability (EICA 1995)	1.98
Enemy inversion, Enemy reduction, Increased susceptibility, New associations	357	Enemy inversion, Enemy reduction, Increased susceptibility, New associations (EI; ERD; IS; NAS 2004)	1.58
Missed mutualism	196	Missed mutualism (MM2006)	1.37
Novel weapons	192	Novel weapons (NW 2004)	1.30
Resource-enemy release (RER 2006)	81	Specialist-generalist, Biotic indirect effects (SG; BID 2004)	1.04
Specialist-generalist, Biotic indirect effects (SG; BID 2004)	67	Enemy of my enemy aka accumulation-of-local-pathogens hypothesis (EE 2006)	0.69
Enemy of my enemy aka accumulation-of-local-pathogens hypothesis (EE 2006)	60	Resource-enemy release (RER 2006)	0.62
Plasticity hypothesis (PH 2006)	41	Shifting defence hypothesis (SDH 2011)	0.58
Shifting defence hypothesis (SDH 2011)	35	Reckless invader aka “boom-bust” (RI 2004)	0.24
Reckless invader aka “boom-bust” (RI 2004)	20		

and IM (invasional meltdown) have stronger external than internal connections, the *niche* and *propagule* cluster do not meet the strong definition here. In general, the centrality of a node in an unweighted graph can be measured by its degree. The analogy in weighted networks is called the *strength* of the node and is defined as the sum of weights of its links. The centrality within a subgraph is then the sum of weights of the node's internal links and can be called its *internal strength* which we use for ranking papers in Table 3.

Discussion

The clusters of networks *M* and *S* are remarkably similar. Two of the four clusters in each network are even identical, namely the concept clusters focused on eco-evolutionary and phylogenetic relationships between non-native and resident species (*Darwin's cluster*) and the concept cluster focused on biotic resistance of ecosystems against non-native species (*resistance cluster)*. Comparing these two networks further, one can see that the other two concept clusters differ just in the membership of PH, the plasticity hypothesis. In one case (*M*-network), PH is in the concept cluster focused on species relationships (*enemy cluster*). In the other case (*S*-network), PH is a member of the concept cluster focused on introduction and species traits (*propagule cluster*).

What are the implications from the networks?

The networks visualize how invasion biologists have seen their research field during the last two decades. Essentially, the networks suggest four broad themes that are represented by the four clusters. One core idea comes from evolutionary biology; it highlights the importance of eco-evolutionary relationships between non-native and resident species, and the capability of species to adapt to new environments (evolutionary perspective, *Darwin's cluster*). A second core idea is the possibility that ecosystems can be resistant, or not, against non-native species based on their characteristics (ecosystem perspective, *resistance cluster*). A third core idea is that species interactions such as host-parasite or predator-prey interactions (including the loss of such interactions in the exotic environment, i.e., enemy release) are very important for understanding biological invasions (species-interactions perspective, *enemy cluster*). Finally, the most recent core idea is that human action is principally influencing biological invasions, which can thus only be understood by studies bridging different research fields (Richardson and Pyšek 2008; Kueffer 2017) (interdisciplinary perspective; *propagule cluster*). Following this line of thought, the discipline of invasion biology is now sometimes called *invasion science*, reflecting that it is not simply a biological subdiscipline but stretches towards other disciplines including social sciences and economics (Richardson and Ricciardi 2013).

Strongly connected hypothesis pairs

Some of the hypotheses in our networks are particularly strongly connected. In this section, we highlight one strongly connected hypothesis pair for each of the four clusters, and outline whether these connections are reasonable.

In *Darwin's cluster*, the two hypotheses adaptation (ADP) and Darwin's naturalization hypothesis (DN) are very strongly connected. The two key publications for these hypotheses included in Table 1 were published at roughly the same time (2001 and 2002). However, DN has its origin in the mid-19th century in what is probably biology's most famous publication of all times (Darwin 1859). As Darwin's book is mainly cited for other reasons than DN, we used another publication as the key paper for DN. The main reason for the strong connection between the hypotheses DN and ADP based on their co-citation in so many papers might be that both hypotheses are contradicting each other (Table 1) and are jointly called *Darwin's naturalization conundrum* (Diez et al. 2008).

In the *resistance cluster*, there is a particularly strong connection between biotic resistance (BR) and the disturbance hypothesis (DS). These two hypotheses are in fact logically linked. According to DS, the invasion success of non-native species is higher in highly disturbed than in relatively undisturbed ecosystems (Table 1). In other terms, highly disturbed ecosystems show lower resistance against non-native species than relatively undisturbed ecosystems. Thus, both hypotheses focus on the resistance of ecosystems against non-native species; BR does so with a focus on biodiversity, and DS with a focus on disturbance (Jeschke and Heger 2018). Another link between the two hypotheses is that disturbance can reduce biodiversity.

In the *propagule cluster*, the propagule pressure hypothesis (PP) is very strongly connected to global competition (GC). The latter hypothesis is actually based on PP (Catford et al. 2009), which explains that these concepts are often jointly cited.

Finally in the *enemy cluster*, the enemy release hypothesis (ER) and EICA hypothesis are particularly strongly connected. This can also be easily explained, as EICA uses enemy release as an underlying assumption (Table 1).

Which network is the better map?

Although the clusters of the two networks are very similar, the better map is in our opinion the *M*-network. This is due to the following two reasons. First, the *M*-network has 25% less edges compared to the *S*-network which results in a clearer picture. Second, the clusters in the *M*-network are better separated from each other than in the *S*-network; all clusters in the *M*-network are communities in the strong sense, but this is only true for two clusters in the *S*-network.

Comparing the network to previous works

In comparison to the other two networks published by Enders and Jeschke (2018) and Enders et al. (2018), the networks of this publication are way clearer. Compared with the similarity-dissimilarity network in Enders et al. (2018), which was created based on an online survey, the *M*- and *S*-networks have fewer connections and clearer, distinct clusters. Apparently, the survey participants had different views on the relationships between invasion hypotheses, possibly because invasion biology has so many hypotheses now that it is hard for researchers to know them all; the similarity-dissimilarity network in Enders et al. (2018) used direct responses given by the survey participants when being asked for hypothesis pairs how similar they are. If participants often simply guessed the similarity of hypothesis pairs, one would expect a random network to emerge from the answers, and this is what Enders et al. (2018) found. This problem was circumvented for two other networks in Enders et al. (2018), joint-mentions networks A and B, which are only based on hypotheses that the survey participants indicated to know best. These networks are clearer than the similarity-dissimilarity network; however, they do not seem to be as useful maps as the networks *M* and *S* presented here. They are not as clear, there clusters have a lower modularity (ca 0.25 for both networks; Enders et al. 2018), and their clusters are not communities in the strong sense.

The network in Enders and Jeschke (2018), which was created by traits of the concepts and hypotheses, has three clusters consisting of concepts with a focus on (i) human interference, (ii) mutualisms, and (iii) enemies (predators or parasites). The modularity is relatively high (ca 0.4) but still lower than for the two networks shown here. Also, the clusters are not communities in the strong sense. This network also seems to be less suitable to serve as a map of the field than the networks shown here, particularly the *M*-network.

Conclusions and outlook

The co-citation approach has proven useful to construct conceptual maps of the field of invasion biology. These maps, particularly the *M*-network, are clearer than previous maps created with other approaches. Efforts to create such conceptual maps that highlight relationships between major concepts within a research field are currently limited. In fact, we are unaware of other attempts to create such maps. This lack of conceptual maps means that researchers lack navigation tools which would help them identify where their work is located within a given research field such as invasion biology. The results of a recent online survey among >350 invasion biologists suggest that the participants lack a "joint vision how invasion hypotheses are related to each other" (Enders et al. 2018). This resembles the situation that invasion biologists lack a common map of the field, which also implies that they do not know where their own work is located in comparison to other studies in the field. The utility of conceptual maps and other navigation tools for research fields thus seems obvious, and it is of course not restricted to invasion biology. Such maps can be provided as interactive visualization tools (https://www.hi-knowledge.org, Jeschke et al. 2018).

But the conceptual maps constructed for this study are early steps on the way towards advanced navigation tools. An important next step would be to allow for concepts and hypotheses to be included in more than one cluster, so that they can take the role of cluster-connecting concepts. To take this next step, a cluster-finding-algorithm that allows overlapping communities should be considered. Furthermore, we have thus far applied three different approaches to create conceptual maps. Other approaches can be imagined as well, for example based on a Delphi-approach in which a group of experts follows multiple iterative steps to create a consensus map. Further work should also involve the expansion of the network to include maps of related fields. In this way, a larger map, or atlas of science (see also Börner 2010, 2015; Kitcher 2011) can be generated that highlights linkages between fields by way of shared broader concepts, such as diversity, stability or the ecological niche (Jeschke 2014). Such a larger atlas of science will undoubtedly foster inter- and transdisciplinary collaboration.

Data accessibility

The R script underlying this study is available via Dryad (https://doi.org/10.5061/dryad.d2q07t6).

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RESEARCH ARTICLE

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Unravelling the origin and introduction pattern of the tropical species *Paracaprella pusilla* Mayer, 1890 (Crustacea, Amphipoda, Caprellidae) in temperate European waters: first molecular insights from a spatial and temporal perspective

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Abstract

Paracaprella pusilla Mayer, 1890 is a tropical caprellid species recently introduced to the Eastern Atlantic coast of the Iberian Peninsula and the Mediterranean Sea. In this study, we used direct sequencing of mitochondrial (COI and 16S) and nuclear (28S and ITS) genes to compare genetic differences in presumed native and introduced populations in order to infer its introduction pattern and to shed light on the native range of this species. The temporal pattern of genetic diversity at the westernmost limit of the geographic range of *P. pusilla* in Europe (the Atlantic coast of southern Spain) over an eight-year period was also investigated. Our results confirm *P. pusilla* as a neocosmopolitan species and suggest that the species is native to the Atlantic coast of Central and South America. *Paracaprella pusilla* seems to have been introduced into European waters from multiple introduction pathways and source populations, which are likely to include populations from coastal waters of Brazil. Multiple introduction pathways may have been involved, with the most important being commercial shipping through the Strait of Gibraltar. While this tropical species appears to be expanding in the Mediterranean, populations from the westernmost limit of its geographic range in Europe showed a temporal instability. This study constitutes the first molecular approach focused on this species, but it is also the first study of temporal change in genetic diversity of any introduced marine amphipod. Additional intensive sampling of this species, including both native and non-native populations, and detailed temporal studies are still necessary to properly understand how genetic diversity influences the introduction and survival of *P. pusilla* in invaded areas.

Keywords
Caprellid amphipod; founder effect; genetic diversity; global change; multiple introduction pathways; population genetics; temporal fluctuations.

Introduction

Non-indigenous species (NIS) are a fundamental component of global change and are currently considered one of the most important drivers of biodiversity alteration in marine ecosystems worldwide (Bax et al. 2003; Molnar et al. 2008). Some NIS successfully establish themselves, form self-sustaining populations, and spread into new locations, becoming invasive and causing both significant ecological and economic impacts (Molnar et al. 2008). They may out-compete native species and alter community structure and ecosystem processes. They may also threaten ecosystem services, which may result in significant economic losses in fisheries, aquaculture, and tourism sectors (Davidson et al. 2015; Ojaveer et al. 2015; Katsanevakis et al. 2016).

Marine organisms have been spread by human-mediated transport long before the first comprehensive biological studies were carried out (Carlton 1999, 2003). However, the ever-increasing magnitude and efficiency of global maritime trade and associated transported vectors, as well as rising seawater temperatures associated with global climate change, are drastically increasing the spread of NIS (Ruiz et al. 1997; Carlton and Cohen 2003; Katsanevakis et al. 2013; Booth et al. 2017).

Europe, where approximately 1500 NIS have been introduced, is the major recipient of marine NIS worldwide (Katsanevakis et al. 2014; AquaNIS 2015; Tsiamis et al. 2018). Consequently, legislation mostly rooted in the Marine Strategy Framework Directive (MSFD) (EC 2008) and the Biodiversity Strategy (EC 2014) has been adopted to deal with NIS and, thus, protect, conserve, or enhance marine ecosystems. These strategies aim to mitigate or reverse the impacts of existing NIS and prevent future introduction and the establishment of new ones by identifying and managing introduction pathways, among other things. In this regard, genetic data have been recognized as a powerful and useful tool (Holland 2000; Geller et al. 2010; Rius et al. 2015). The study of the genetic structure and degree of gene flow within and between native and non-native populations specifically provides crucial insights into the pattern of introduction, colonization, and spread of introduced taxa (Geller et al. 2010; Rius et al. 2015). Such studies help to determine source populations (Rius et al. 2015) and provide insights into the invasive potential of species (Roman and Darling 2007), and, thus, can lead to a better understanding of the mechanisms and dynamics underlying introduction and invasion. Such information can be used to prioritize management strategies, to prevent further introduction events, and to assess the abundance and status of NIS.

Nonetheless, most genetic studies on NIS have focused on terrestrial and freshwater organisms (Lowry et al. 2013; Sherman et al. 2016; Viard et al. 2016), likely due to the logistics of sampling in the marine environment, which make it difficult to obtain reliable neutral population estimates (Sherman et al. 2016). Therefore, more research is needed to improve our knowledge of marine NIS and better understand the patterns of their introduction and invasion. On this subject, hotspots and stepping-stone areas for these species (e.g. marinas, aquaculture installations) and zones of special interest such as marine reserves or NATURA 2000 sites, should be prioritized (Olenin et al. 2010). In European waters, the Mediterranean Sea and the region of the Strait of Gibraltar deserve special attention, as they are considered hotspots for both biodiversity and biological invasions (Drake and Lodge 2004; Molnar et al. 2008; Boudouresque et al. 2017). Indeed, the Mediterranean Sea hosts the highest documented number of marine NIS globally, with around 900 NIS recorded so far (Ulman et al. 2017; Zenetos et al. 2017; Galil et al. 2018). Both the Mediterranean Sea and the Strait of Gibraltar have important shipping links to other areas worldwide, intense aquaculture activity, and are among the most important destinations for tourism worldwide, with many vessels arriving from America and northern Europe as well as from the Indo-Pacific via the Suez Canal (Streftaris et al. 2005; Galil et al. 2017; Tsiamis et al. 2018).

Crustaceans are among the most introduced taxa worldwide (Carlton 2011). In the Mediterranean Sea and nearby areas, they account nearly 20% of all reported NIS (Zenetos et al. 2012; Ulman et al. 2017). Within crustaceans, caprellid amphipods associated with fouling communities of artificial habitats are considered as prime candidates for introduction and establishment in regions where they are not native (Ashton et al. 2010; Ros et al. 2016a). The great abundances that some caprellids attain in these communities and their ability to survive on floating objects and vessel hulls (Thiel et al. 2003; Ashton et al. 2010) make them good models for understanding marine introductions and invasions. Thus, the number of introduced species belonging to this group have been documented worldwide with increasing frequency (e.g. Ros et al. 2016a; Gillon et al. 2017; Marchini and Cardeccia 2017).

Three non-indigenous species of caprellids have been recorded in temperate European seas: *Caprella mutica* Schurin, 1935 (Ashton 2006; Almón et al. 2014), *Caprella scaura* Templeton, 1836 (Sconfietti and Danesi 1996; Martinez and Adarraga 2008), and *Paracaprella pusilla* Mayer, 1890 (Ros and Guerra-García 2012; Ros et al. 2016a). While the dynamics of invasion by *C. mutica* and *C. scaura* have been explored through molecular tools (Ashton et al. 2008; Cabezas et al. 2014), there are no similar studies for *P. pusilla*.

Paracaprella pusilla is a tropical caprellid species first described from Brazil (type locality: Rio de Janeiro) (Mayer 1890). Nonetheless, the native range of this species is not entirely clear (Farrapeira et al. 2011; Rocha et al. 2013). Some authors have considered the Atlantic coast of Central and South America as the most likely native range of this species (Mayer 1903; McCain 1968; Carlton and Eldredge 2009; Rocha et al. 2013). *Paracaprella pusilla* is frequently found in the Caribbean (Carlton and Eldredge 2009), with most records coming from the Gulf of Mexico and the coasts of Venezuela and Colombia (Díaz et al. 2005; Guerra-García 2006; Winfield et al. 2006). However, other authors have considered *P. pusilla* to be cryptogenic, sensu Carlton (1996), in this region (Serejo 1998; Farrapeira et al. 2011). Since its original description, *P. pusilla* has been reported from numerous other tropical and subtropical areas around the world, including both the East and West African coasts, the Indian peninsula, Australia, and Hawaii (see Ros and Guerra-García 2012 and references therein), mainly on fouling communities associated with artificial structures. Today, the known distribution of *P. pusilla* also includes the Pacific coasts of Panama (Ros et al. 2014), Mexico (Alarcón-Ortega et al. 2015), and Costa Rica (Alfaro-Montoya and Ramírez-Alvarado 2018), in addition to temperate European waters. Its first recorded occurrence (2010) in Europe was at Cádiz on the Atlantic coast of southwestern Spain (Ros and Guerra-García 2012). Soon afterwards, this species was first found in the western Mediterranean at Mallorca (2011) and Ibiza in August 2012 (Ros et al. 2013c). In 2014, several individuals of *P. pusilla* were found at Zikim on the southern coast of Israel, the first record of this species in the eastern Mediterranean Sea (Ros et al. 2016a). Most recently, *P. pusilla* has been reported in the central Mediterranean, in the Gulf of Gabès in tidal channels of the Kneiss archipelago (Tunisia) (Fersi et al. 2018). Thus, by its presence in western, central, and eastern regions of the Mediterranean, we suggest that *P. pusilla* might be present more generally throughout the Mediterranean and that it might have been overlooked due to its small size or temporal instability.

Two main pathways have been suggested for the introduction of *P. pusilla* to European waters. Ship fouling is the most probable vector for the introduction and dispersion of this species (Ros and Guerra-García 2012; Ros et al. 2013b, c), either through the Strait of Gibraltar, from source populations in Central and South America, where it is supposedly native, or via the Suez Canal, from the Indo-Pacific. This second alternative is less likely because *P. pusilla* has not yet been found in the Red Sea (Zeina and Guerra-García 2016) and has only been reported once in the Suez Canal (Schellenberg 1928) despite recent studies (see El-Komi 1998; Emara and Belal 2004; Zeina and Guerra-García 2016). These pathways are only assumptions based on historical records of *P. pusilla*. No molecular studies have been conducted to elucidate the introduction and dispersion patterns of this species.

Another question that remains genetically unexplored is whether *P. pusilla* is indeed a cosmopolitan species or if populations across its presumed large range belong to different cryptic species. In the order Amphipoda, molecular evidence supports the existence of cryptic species among widely distributed marine NIS, such as *Ampithoe valida* Smith, 1873 and *Jassa marmorata* Holmes, 1905, two biofouling species introduced to the Northeast Pacific (Pilgrim and Darling 2010). Nevertheless, other NIS are actually widely distributed (no cryptic species); examples include the caprellids *Caprella mutica* and *Caprella scaura* sensu stricto (Ashton et al. 2008; Cabezas et al. 2014). Morphological evidence supports the conspecificity of populations of *P. pusilla* (Ros et al. 2014), but molecular evidence is still needed to confirm *P. pusilla* as a neocosmopolitan species (introduced species that have achieved a widespread distribution through anthropogenic dispersal; sensu Darling and Carlton 2018).

In this study, we analysed the genetic diversity, population structure, and levels of differentiation of populations of *P. pusilla* from its presumed native and introduced distribution ranges. We sequenced mitochondrial and nuclear genes of *P. pusilla* in order to (i) provide the first molecular evidence to verify the conspecificity of populations; (ii) shed light on this species' native range, and (iii) to infer its introduction pattern in temperate European waters, particularly on the Iberian Peninsula. In addition, we analysed the temporal pattern of genetic diversity at Cadiz marina, which is the westernmost limit of the range of *P. pusilla* in Europe, beginning soon after its first detection and for a period of eight years. We use the Cadiz marina as a model for understanding how genetic diversity influences the introduction process of this tropical NIS into new areas where it previously could not survive. This information is crucial to better understanding the initial phases of marine introductions and identifying the factors associated with it. Additionally, this information allows for the better understanding of possible future invasions to other localities on the Atlantic coasts of Europe in the scenario of global warming, and, thus, it provides valuable information for the effective management of introduced species. As far as we know, this is the first study of temporal change in genetic diversity of an introduced marine amphipod.

Methods

Sample collection

Spatial sampling. A total of 230 specimens of *P. pusilla* were collected from 12 localities across its presumed native and introduced geographic ranges, including from the type locality at Rio de Janeiro and the whole of its introduced range in Europe (Table 1). Unfortunately, the Indo-Pacific region was represented only by a single sample from Australia. The greater number of individuals collected at Spanish localities is the result of our continuous monitoring over eight years, when at least two samplings per year were conducted. Samples were collected mostly from fouling communities predominantly comprised of hydroids and macroalgae, attached to floating pontoons, ropes, buoys, and ship hulls. At each locality, individuals were removed by hand and immediately preserved in 96–100% ethanol. In the laboratory, using a stereomicroscope, male individuals (see Guerra-García 2006) were identified as *P. pusilla* (see Mayer 1903: pl. 2, figs 36, 37; Ros et al. 2013c: fig. 2).

To compare the levels of intra- and interspecific genetic diversity, four individuals of the congeneric *Paracaprella tenuis* Mayer, 1903 from Celestún, Mexico (Table 1)

Table 1. *Paracaprella pusilla* sampling information. Sampling localities, location codes, source countries, geographical coordinates, substrata, and year of collection. Data for Cadiz Marina (ESCAD) correspond to the sample used in the spatial analysis.

Locality	Location Code	Country	Coordinates	Habitat	Sampling
Paracaprella pusilla					
Cadiz Marina (Puerto América, Cádiz)	ESCAD	Spain	36°32'29"N, 6°17'61"W	Marina – *Eudendrium* spp.	2010–2017
Puente de Hierro Marina (San Fernando, Cádiz)	ESSFN	Spain	36°29'02"N, 6°10'44"W	Marina – *Eudendrium* sp.	2016
Palma Marina (Baleares)	ESBAL	Spain	39°33'54"N, 2°37'58"E	Marina – *Halocordyle* sp.	2011–2012
Gulf of Gabès (Kneiss Channel)	TNGGB	Tunisia	34°20'46"N, 10°14'44"E	Fine sand	2016
Zikim Beach	ILZIK	Israel	31°36'45"N, 34°30'16"E	Drifting *Bugula neritina*	2014
Trinity Inlet Cairns (Queensland)	AUAUS	Australia	16°57'56"S, 145°47'34"E	Raft	2013
Ilha Cotinga (Paraná)	BRILH	Brazil	25°31'36"S, 48°28'22"W	Submerged artificial substrata	2012
Paranaguá Bay (Paraná)	BRPAB	Brazil	25°30'03"S, 48°31'47"W	Experimental plates	2009
Paranaguá Marina (Paraná)	BRPAR	Brazil	25°30'53"S, 48°29'52"W	Marina – *Eudendrium* sp.	2012
Niteroi (Rio de Janeiro)	BRRIO	Brazil	22°55'42"S, 43°06'36"W	Marina – Hydroids spp.	2012
São Sebastião (São Paulo)	BRSAO	Brazil	23°46'06"S, 45°24'06"W	Marina – *Eudendrium* sp.	2012
Sisal	MXSIS	Mexico	21°40'44"N, 90°03'26"W	Drifting seaweeds on sediment	2010
Paracaprella tenuis					
Celestún	MXCEL	Mexico	20°51'32"N, 90°24'08"W	Drifting seaweeds on sediment	2010
OUTGROUPS					
Caprella liparotensis					
Benalmádena (Málaga)	ESBENA	Spain	36°34'51"N, 04°33'30"W	Intertidal macroalgae	2014
Caprella danilevskii					
Al-Hoceima	MAAHO	Morocco	35°15'04"N, 03°55'09"E	Intertidal macroalgae	2013

were also included. *Paracaprella tenuis* is very similar to *P. pusilla* (see morphological characters by McCain 1968; Winfield and Ortiz 2013: table 1) and these species occur in sympatry in the northern Gulf of Mexico (Foster et al. 2004).

Temporal sampling. *Paracaprella pusilla* was first recorded in Europe in September 2010 on a floating pontoon at Cadiz marina, southern Spain, during a survey of peracarid crustaceans from harbours along the Strait of Gibraltar (Ros and Guerra-García 2012). At this locality, *P. pusilla* is associated with *Eudendrium* sp., a hydroid and a common component of fouling communities; these species seem to have a mutualistic relationship (Ros and Guerra-García 2012; Ros et al. 2013a). Samples of the Cadiz population were collected annually from 2010 to 2017 (Table 1), mostly during summer or early autumn, when caprellid abundance was generally greatest. Individuals of *P. pusilla* were removed by hand from samples of *Eudendrium* collected from the sides of floating pontoons, near the water surface. Seventy-five specimens were collected from this site and immediately preserved in 96–100% ethanol.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from gnathopods, pereopods, antennae and gills along one side of the body of each specimen sampled. We used the commercial kit PureLink Genomic DNA Mini Kit (Invitrogen, UK) according to the manufacturer's protocol. The DNA was eluted in 120 µl of elution buffer and stored at –20 °C.

Fragments of two mitochondrial (COI and 16S rRNA) and two nuclear (28SrRNA and ITS) genes were amplified by polymerase chain reaction (PCR), the latter two genes only for a subset of representative individuals of each population. PCR amplifications consisted of 25 µl reaction volumes containing 3 µl of template DNA, 10× $MgCl_2$-free buffer (Invitrogen, UK), 3 mM (for COI gene)/2.5 mM (for 16S, 28S and ITS genes) $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer, 0.1 µg µl^{-1} Bovine Serum Albumin (BSA, Promega, Madison, WI), 0.3 U Platinum Taq DNA polymerase (Invitrogen, UK), and double-distilled H_2O to volume. Primers for amplification and PCR conditions are listed in Table 2.

PCR product purification and unidirectional or bidirectional Sanger sequencing were provided by a commercial company (GENEWIZ, London, UK).

Table 2. Primers used for amplification and PCR conditions used in the present study.

Primer	Sequence (5'-3')	Source	PCR conditions
COI			
jgLCO1490	TITCIACIAAYCAYAARGAYATTGG	Geller et al. (2013)	94 °C (4'); [x40] 94 °C (45''), 45 °C (50''), 72 °C (1'); 72 °C (10')
jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA		
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	
HCO1490	TAAACTTCAGGGTGACCAAAAAATCA		
16S rRNA			
16STf	GGTAWHYTRACYGTGCTAAG	Macdonald et al. (2005)	94 °C (2.30'); [x36] 94 °C (40''), 54 °C (40''), 65 °C (1.20'); 65 °C (8')
16Sbr	CCGGTTTGAACTCAGATCATGT	Palumbi et al. (1991)	
28S rRNA			
28S rd1a	CCCSCGTAAYTTAGGCATAT	Edgecombe and Giribet (2006)	94 °C (4'); [x40] 94 °C (20''), 58 °C (1'), 72 °C (2'); 72 °C (10')
28Sb	TCGGAAGGAACCAGCTAC	Whiting (2002)	
28SDKF	GATCGGACGAGATTACCCGCTGAA	Strong et al. (2011)	
LSU1600R	AGCGCCATCCATTTTCAGG	Williams et al. (2003)	
ITS			
ITS1F	CACACCGCCCGTCGCTACTACCGAT	Chu et al. (2001)	94 °C (1.30'); [x33] 94 °C (20''), 56.8 °C (30''), 72 °C (30''); 72 °C (5')
ITS1R	GCGGCAATGTGCATTCGACATGTGA		

Sequence analysis

The resulting sequences were checked and edited using SEQUENCHER version 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Mitochondrial COI sequences were translated into amino acids to search for stop codons that are indicative of the presence of pseudogenes. All sequences were thereafter deposited in GenBank (Suppl. material 1, Table S1).

For mitochondrial (COI and 16S) and ITS genes, all sequences were aligned using MUSCLE (Edgar 2004) as implemented in MEGA version 7 (Kumar et al. 2016). Sequences of both mitochondrial genes were subsequently concatenated using the APE package (Paradis et al. 2004) in RStudio (RStudio Team 2016). For 28S gene, sequences were aligned using the MAFFT algorithm (Katoh and Standley 2013) and highly variable regions were eliminated from the analyses using GBLOCKS (Castresana 2000) with default parameters and allowing all gap positions. Uncorrected pairwise distances among haplotypes were calculated using MEGA version 7 (Kumar et al. 2016).

Spatial analysis

Phylogenetic reconstruction. Phylogenetic relationships were estimated using two model-based methods of phylogenetic inference to verify whether alternative topologies were supported by different tree-building approaches: Bayesian inference (BI) in MrBayes version 3.2.6 (Ronquist et al. 2012) and maximum likelihood (ML) in RAxML version 7.2.8 (Stamatakis 2008). These analyses were carried out for three sequence datasets: one using the concatenated sequences of the mitochondrial genes (COI + 16S), other using sequences of the nuclear 28S ribosomal gene, and the last using sequences of the nuclear ITS gene. Analyses were conducted using data partitions by codon (1+2+3) for the mitochondrial COI gene, to minimize saturation effects of codon positions on phylogenetic reconstructions (Salemi 2009) and to account for different rates of evolution of each one (Pond et al. 2009). Only one individual (or sequence) per haplotype was included in the phylogenetic analyses. *Caprella liparotensis* Haller, 1879 and *Caprella danilevskii* Czerniavski, 1868 were used as outgroups (Table 1). The best-fit model of sequence evolution for the three datasets was estimated using PartitionFinder version 2.1.1 (Lanfear et al. 2016). According to the corrected Akaike Information Criterion (AICc) (Akaike 1974), the best models for the mitochondrial concatenated dataset were GTR+I (1st partition), GTR (2nd partition), GTR+G (3rd partition), and GTR+G (4th partition = 16S), and for the 28S and the ITS genes, the models GTR+I and GTR+G were selected, respectively. For BI analyses, two independent runs, of four chains each, were conducted for 2×10^7 generations (runs converged with average standard deviation of the split frequencies below 0.01). Trees and parameters were sampled every 1000 generations, with the heating parameter set to 0.25. The consensus (majority-rule) tree was estimated combining results from duplicated analyses, after discarding 25% of total samples as burn-in, determined from plotting log-likelihood values against generation time in Traver version 1.7.1 (Rambaut et al. 2018). For ML analyses, phylogenetic tree was calculated using the GTRGAMMAI model for the mitochondrial dataset, GTRCATI for the 28S gene, and GTRGAMMA for the ITS gene, and bootstrap supports were calculated using 1000 replicates. Consensus tree inferred for each molecular dataset was visualized and rooted using FigTree version 1.4.3 (Rambaut 2017).

Furthermore, relationships among mitochondrial haplotypes (using the concatenated dataset) were examined via a haplotype network using statistical parsimony method (Templeton et al. 1992) in TCS version 1.21 (Clement et al. 2000) with a 95% connection limit. The network was plotted with tcsBU (Santos et al. 2016).

Estimates of genetic diversity and population structure. Two measures of mtDNA diversity, haplotype (*Hd*, Nei 1987) and nucleotide diversity (π, Nei 1987), were estimated for each *P. pusilla* locality and region, using DnaSP version 6 (Rozas et al. 2017). Three distinct regions were considered: Northeast Atlantic + Mediterranean, South Pacific, and Western Atlantic (presumed native region). The single individual from Israel (ILZIK) was excluded from the analysis.

The genetic differentiation among populations was determined by means of the statistics F_{ST} (Weir and Cockerham 1984) with Arlequin version 3.5.1.2 (Excoffier and Lischer 2010), using the pairwise differences distance method. Pairwise F_{ST} values were calculated for the mitochondrial dataset, excluding the population with less than three individuals (i.e. ILZIK). Statistical significance was assessed through 10000 permutations, and a multidimensional scaling (MDS) analysis was performed on the matrix of F_{ST} values for a graphical depiction of the structure using TIBCO STATISTICA version 13 (TIBCO Software Inc., CA, USA). Additionally, a hierarchical analysis of molecular variance (AMOVA, Excoffier et al. 1992) was conducted in Arlequin version 3.5.1.2 (Excoffier and Lischer 2010) to study the distribution of genetic variability between presumed native and introduced *P. pusilla* populations and to explore differentiation across geographic locations. In this regard, two groups were used for the AMOVA tests: (i) presumed native (Brazil + Mexico) vs non-native (Spain + Tunisia + Australia), and (ii) regions (Northeast Atlantic + Mediterranean; South Pacific; Western Atlantic). Statistical significance of variance components was tested with 16000 permutations.

Finally, to test if the selection of demographic events (population expansion or contraction) affected the genetic structure of non-native and potentially native populations, neutrality tests (Tajima's D, Fu's FS and Ramos-Onsis and Rozas' R_2) (Fu 1997; Tajima 1989; Rozas and Ramos-Onsins 2002) and mismatch distribution were performed for the mitochondrial dataset. Neutrality tests provide trends with respect to equilibrium and non-equilibrium conditions and indicate recent population expansion when the null hypothesis of neutrality is rejected due to significant negative values. They were assessed for each region with the statistical significance obtained by 10000 coalescent simulations. The distribution of frequencies of observed numbers of differences between pairs of haplotypes for each region is shown in the mismatch distribution. It uses tree shape to provide a rough estimate of population expansion or contraction because of a bottleneck. Populations that have experienced rapid demographic growth in the recent past exhibit unimodal distributions, whereas populations that have been constant over time (demographic equilibrium) have bimodal or multimodal distributions (Rogers and Harpending 1992; Haydar et al. 2011). To test the goodness-of-fit between the observed and the expected distributions under the sudden expansion model, the sum of squared deviations (SSD) (Schneider and Excoffier 1999) and Harpending's raggedness index (Rg) (Harpending 1994) were also computed using 10000 bootstrap replicates. DnaSP version 6 (Rozas et al. 2017) was used to calculate R_2 statistic, and the remaining estimates and respective significance tests were obtained with Arlequin version 3.5.1.2 (Excoffier and Lischer 2010). In all analyses, localities with fewer than three individuals (i.e. ILZIK) were excluded.

Temporal monitoring

In the Cadiz marina (ESCAD) population, genetic diversity over time was assessed by estimating the haplotype (*Hd*) and nucleotide (π) diversity (Nei 1987) for each year sampled using DnaSP version 6 (Rozas et al. 2017). Frequencies of haplotypes per year were also calculated with this program. In addition, to test whether variation in genetic diversity (haplotype diversity) was linearly related to time (in years), a linear regression analysis was performed using the ggplot2 package (Wickham 2016) in RStudio (RStudio Team 2016).

Estimates of population differentiation over time were obtained from pairwise F_{ST} calculations for the mitochondrial dataset, and neutrality tests (Tajima's D, Fu's FS and Ramos-Onsis and Rozas' R_2) were also estimated. All these analyses were conducted as described for the spatial analysis. The MDS analysis based on the matrix of F_{ST} values was performed together with the data from the spatial analysis.

Results

Sequence variation

The mitochondrial markers COI and 16S rRNA were successfully amplified for 236 caprellid individuals: 230 *Paracaprella pusilla*, four *P. tenuis*, and the outgroups *Caprella liparotensis* and *C. danilevskii* (Suppl. material 1, Table S1). Overall, 44 haplotypes were observed: 39 for *P. pusilla*, three for *P. tenuis*, and one for each of the outgroup species (Table 3). The complete alignment of the COI dataset had a total length of 612 bp. No insertions or deletions were detected in any of the sequences, and when they were translated into proteins, no stop codons were found. However, for the 16SrRNA (alignment of 408 bp), some indels were identified. Most of these correspond to insertions or deletions in sequences for *P. tenuis* or *Caprella* spp. Interestingly, among *P. pusilla*, a one bp insertion of a thymine (T) was observed at position 45 in six individuals from Australia and Brazil (all corresponding to the haplotype H25). The alignment of the concatenated dataset of these two genes (COI + 16S) had a total of 1022 bp.

Table 3. Genetic diversity of *Paracaprella pusilla* for each sampling site and region. Number of individuals per location (N), number of haplotypes (H), haplotype codes (private haplotypes indicated in **bold**), haplotype diversity (*Hd*) and nucleotide diversity (*π*).

Region	Population	N	H	Haplotype codes	*Hd*	*π*
Northeast Atlantic + Mediterranean	ESCAD	75	9	H1, H2, H3, H4, **H5**, H6, H7, **H8**, H9	0.764	0.00598
	ESSFN	25	8	H1, H2, H3, H4, H7, H9, **H10**, **H11**	0.713	0.00366
	ESBAL	53	10	H2, H6, **H12**, **H13**, **H14**, **H15**, **H16**, **H17**, **H18**, H19	0.777	0.00240
	TNGGB	3	2	H20, **H21**	0.667	0.00066
	ILZIK	1	1	H2	–	–
	Total	**157**	**21**		**0.849**	**0.00460**
South Pacific	AUAUS	15	6	H2, H19, H22, **H23**, **H24**, H25	0.790	0.00220
	Total	**15**	**6**		**0.790**	**0.00220**
Western Atlantic (presumed native)	BRILH	10	5	H2, H25, **H26**, H27, H28	0.756	0.00305
	BRPAB	11	5	H22, H25, **H29**, **H30**, **H31**	0.782	0.00456
	BRPAR	7	4	H2, H20, H28, **H32**	0.810	0.00104
	BRRIO	6	4	H2, H27, **H33**, **H34**	0.800	0.00125
	BRSAO	9	4	H2, H25, **H35**, **H36**	0.778	0.00269
	MXSIS	15	4	H2, **H37**, **H38**, **H39**	0.600	0.00151
	Total	**58**	**18**		**0.877**	**0.00310**
	TOTAL	**230**	**39**		**0.879**	**0.00418**

The nuclear marker 28S was amplified for 60 *P. pusilla* individuals and the two outgroups species (Suppl. material 1, Table S1). Unfortunately, we were not able to amplify this gene for any individuals of *P. tenuis*. The total alignment length was 1265 bp, but only 1135 bp were selected using the software GBLOCKS. Some insertions and deletions were found, most of them distinguishing between *P. pusilla* and the outgroup species. Among *P. pusilla* sequences, a lack of sequence variation was observed: only two haplotypes, differing by the presence of an indel at position 443–444 of the alignment, were retrieved for the 60 individuals sequenced.

Finally, the alignment of the nuclear ITS marker had a total of 518 bp and included 73 *P. pusilla* and four *P. tenuis* individuals, plus the two outgroup species (Suppl. material 1, Table S1). Some indels were found between *P. pusilla* and the other species. However, all *P. pusilla* sequences were identical.

Spatial analysis

Phylogenetic reconstruction. Phylogenetic analyses of the mitochondrial dataset using the two different approaches (ML and BI) rendered trees with similar overall topologies, with main clades receiving high bootstrap or posterior probabilities support (Suppl. material 2, Fig. S1). All analyses revealed that *P. pusilla* and *P. tenuis* are monophyletic and formed highly supported clades. Within *P. pusilla*, no clear genetic structure was apparent and all haplotypes from the presumed native and non-native ranges appeared mixed, matching the results from the haplotype network (see below).

Nevertheless, haplotypes 6 and 7 appeared a little more differentiated from the remaining haplotypes. Pairwise divergence (uncorrected *p* distances) between most *P. pusilla* haplotypes was small, not exceeding 1.1%, with the exception of H6 and H7, which differed from other haplotypes by 2.0–2.4% (Suppl. material 3, Table S2). Interspecific divergence within the genus *Paracaprella* were much larger that intraspecific variation, ranging from 16.6 to 17.9%, which is similar to the values found between the two *Caprella* species included in this study (21.5%) (Suppl. material 3, Table S2). For the 28S gene, the ML and BI analysis produced a tree with identical topologies. *Paracaprella pusilla* was found to be monophyletic in both analyses (Suppl. material 5, Fig. S2A). Sequence divergence between them was 0%, whereas divergence between *Caprella* species was 4.5%. Divergence between the two genera exceeded 25%. Finally, for the ITS, the ML and BI analyses also rendered trees with identical topologies (Suppl. material 5, Fig. S2B). For this gene, sequence divergence between *P. pusilla* and *P. tenuis* was 16.9%, a higher value than that found between *Caprella* species (10.7%).

The haplotype network reconstruction for all sequenced mtDNA data retrieved two separate networks that could not be connected using the 95% parsimony connection limit (Fig. 1). For the first network, a star-like phylogeny was observed, with one very common haplotype surrounded by several low-frequency and some medium-frequency haplotypes with a maximum distance of eight mutation steps (corresponding to the haplotype H25). Interestingly, this most different haplotype corresponds to those individuals from Australia and Brazil that presented a one bp insertion at position 45 of the alignment. The central haplotype (H2) accounts for ~30% of *P. pusilla* individuals sequenced and was detected at all locations except the Gulf of Gabès, Tunisia (TNGGB; only three individuals sequenced) and Paranaguá Bay (BRPAB) (Table 3). The remaining haplotypes found in Europe were within one to four point mutation steps from the central haplotype, those from Australia differed by one to eight steps (Fig. 1). The second network included only two haplotypes (H6 and H7) separated by seven mutation steps; these were detected in 10 individuals of Cadiz (ESCAD and ESSFN) and Baleares (ESBAL) populations (Fig. 1).

Genetic diversity and population structure. The spatial distribution of the 39 mitochondrial haplotypes of *P. pusilla* did not show any clear pattern (Table 3; Fig. 2). Private haplotypes were present in almost all populations. Eighteen haplotypes were identified in the presumed native range at the Atlantic coast of America, twelve of them were private (Table 3; Fig. 2). Among populations from other regions that are considered non-native (Europe and Australia), 25 haplotypes (14 private) were identified and only four of them (H2, H20, H22, and H25) were shared with the presumed native locations. Only H2 was shared between European non-native populations and the presumed native ones. Eight haplotypes (H1–H4, H6, H7, H9, and H19) were shared among non-native populations, most of them between ESCAD and ESSFN (i.e. Cadiz populations). Among these haplotypes, only one (H2) was present in almost all non-native populations (except TNGGB), but also in all presumed native locations except BRPAB (Table 3; Fig. 2).

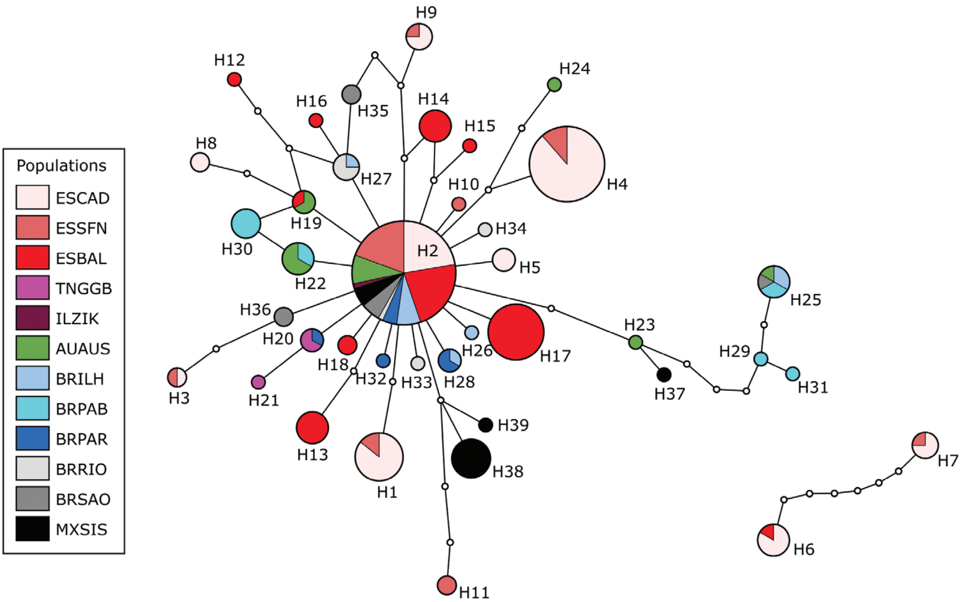


Figure 1. Mitochondrial (COI+16S) haplotype network of *Paracaprella pusilla* from its presumed native and non-native range. Haplotypes 6 and 7, corresponding with 10 individuals of Cadiz (ESCAD and ESSFN) and Baleares (ESBAL) populations, were grouped in an independent network. This network could not be connected using the 95% parsimony connection limit to the main haplotype network which includes most of the haplotypes found in *P. pusilla*. Haplotype circles are proportional to haplotype frequency and numbers represent haplotype identities (Table 3). Non-observed haplotypes (extinct or unsampled haplotypes) are represented by small white circles. Each line connecting haplotypes represents a single mutational step.

Overall, haplotype (*Hd* = 0.879) and nucleotide (π = 0.00418) diversities were high (Table 3). Both presumed native and non-native populations showed similar values of haplotype diversity (Table 3). Highest haplotype diversities were found for the presumed native populations of Paranaguá marina (BRPAR) and Niteroi (BRRIO), and the supposedly non-native population of Australia (AUAUS) (*Hd* = 0.810, 0.800 and 0.790, respectively). The lowest haplotype diversity was found for the presumed native population of Mexico (MXSIS) (*Hd* = 0.600; Table 3). Among Eastern Atlantic and Mediterranean populations, Palma marina (ESBAL) displayed the highest value of haplotype diversity (*Hd* = 0.879) and TNGGB the lowest (*Hd* = 0.667; Table 3). Nucleotide diversity ranged from 0.00066 in TNGGB to 0.00598 in ESCAD.

The estimates of pairwise F_{ST} values showed mostly low and intermediate levels of divergence between populations, with significant values ranging from 0.067 (ESCAD-AUAUS) to 0.538 (TNGGB-MXSIS) (Table 4). Despite the great geographic distances, F_{ST} values between presumed native and non-native populations were not high. However, they revealed that the Gulf of Gabès (TNGGB), Paranaguá Bay (BRPAB), and Sisal (MXSIS) were genetically differentiated from most other populations (Table

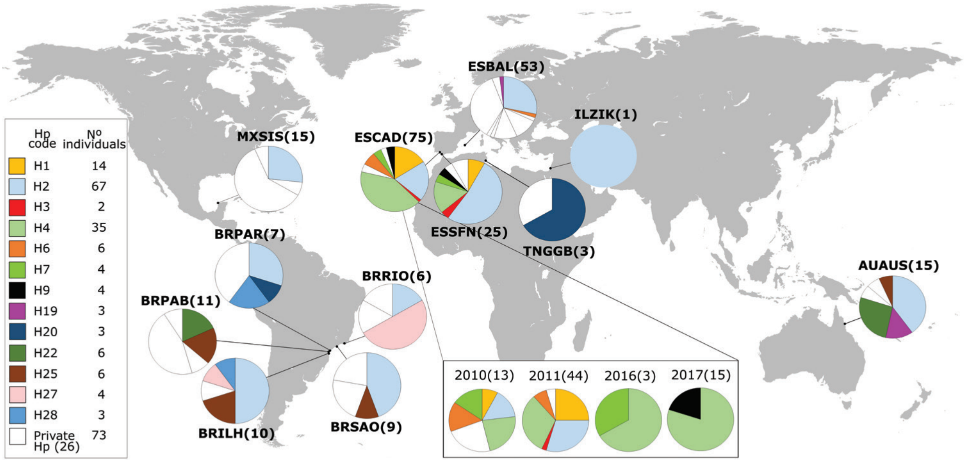


Figure 2. Geographical distribution of the 39 mtDNA haplotypes (Hp) of *Paracaprella pusilla* in the populations sampled. Each site is represented by a pie chart showing population composition and relative haplotype frequency. Number of analysed individuals per population appears in brackets. White-shaded areas are the cumulative proportion of private haplotypes per location. Sites are coded as in Tables 1 and 2. The legend gives information about the existing haplotypes across all locations and the number of individuals carrying each haplotype. For the Cadiz marina (ESCAD) population, the change in haplotype frequency is shown in the four years when the species was recorded.

Table 4. Pairwise F_{ST} values between populations of *Paracaprella pusilla*, based on mtDNA COI+16S sequences. Significant values ($p < 0.05$) are indicated with an asterisk.

	ESCAD	ESSFN	ESBAL	TNGGB	AUAUS	BRILH	BRPAB	BRPAR	BRRIO	BRSAO
ESCAD										
ESSFN	0.019									
ESBAL	0.125*	0.072*								
TNGGB	0.105	0.142	0.275							
AUAUS	0.067*	0.021	0.088*	0.282*						
BRILH	0.078	0.044	0.122*	0.219*	0.006					
BRPAB	0.192*	0.228*	0.335*	0.286*	0.122	0.088				
BRPAR	0.049	–0.009	0.058	0.431*	0.394	0.044	0.255*			
BRRIO	0.058	0.026	0.095	0.511*	0.099	0.070	0.260*	0.179*		
BRSAO	0.059	0.012	0.089*	0.244*	0.013	–0.046	0.156	0.044	0.005	
MXSIS	0.187*	0.195*	0.293*	0.538*	0.297*	0.290*	0.401*	0.367*	0.397*	0.296*

4), although in the case of the Gulf of Gabès population this might be an effect of a low sample size ($N = 3$). These patterns are reflected in the MDS plot, which did not show any clear separation between non-native and presumed native populations, but TNGGB, BRPAB and MXSIS were slightly separated from the others (Fig. 3). Hierarchical AMOVA tests revealed significant genetic differences within populations, and among populations within groups at all geographical levels (native vs non-native, and

Table 5. AMOVA tests. Results of the AMOVA tests comparing variation in mitochondrial sequences of *Paracaprella pusilla* grouped at two geographical levels: (A) presumed native vs non-native, and (B) regions. Significance at $p < 0.05$ (*) and at $p < 0.0001$ (**). Statistical probabilities derived from 16000 permutations.

Group	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
A Presumed native vs non-native	Between groups	1	13.062	0.067	3.02 (F_{CT} = 0.030)
	Among populations w/in groups	9	57.520	0.245	11.01 (F_{SC} = 0.114*)
	Within populations	218	417.707	1.916	85.97 (F_{ST} = 0.140**)
	Total	228	488.288	2.229	
B Regions	Among groups	2	17.036	0.024	–5.30 (F_{CT} = 0.011)
	Among populations w/in groups	8	53.546	0.263	23.84 (F_{SC} = 0.121*)
	Within populations	218	417.707	1.916	81.46 (F_{ST} = 0.130**)
	Total	228	488.288	2.203	

regions) (Table 5). Intrapopulation variance explained most (over 80%) of the genetic variation found in *P. pusilla* (Table 5).

Neutrality tests, Tajima's D, Fu's FS and Ramos-Onsis and Rozas' R_2, were negative for all regions but not statistically significant (Table 6); note that according to Fu (1997), FS statistic should be considered as significant if its *p*-value is less than 0.02. Additionally, the observed mismatch distribution was nearly bimodal for all regions (Fig. 4), which, thus, disproves the sudden expansion model and suggestes possible diminishing or structured population sizes. Regarding the sum of the square deviations (SSD), statistically significant differences were observed ($p < 0.05$) in presumed native populations and in the introduced Australian population (Table 6), which further support no recent population expansion. Nevertheless, these results contrast with the non-significant values of the Harpending's raggedness index (Rg) (Table 6), which indicated that a recent population expansion may have occurred in these populations. In addition, both SSD and non-significant values of Rg suggested goodness of fit between the observed and the expected distributions in East-Atlantic and Mediterranean introduced populations (Table 6; Fig. 4a), and, thus, the null hypothesis of recent population expansion should not be totally rejected.

Temporal monitoring

Paracaprella pusilla was monitored in Cadiz marina (ESCAD) soon after its first detection and for a period of eight years (2010–2017). However, the species was not found during the surveys carried out from 2012 to 2015. Therefore, we considered only four years (2010, 2011, 2016 and 2017) in our study.

Nine haplotypes (same as in the spatial study; Table 3), were obtained from the 75 individuals sequenced (Suppl. material 4, Table S3; Fig. 2). Interestingly, haplotype

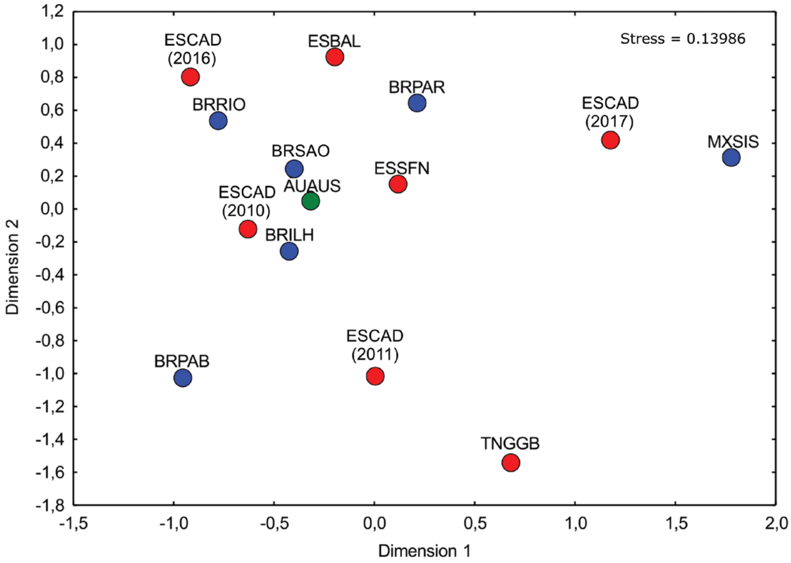


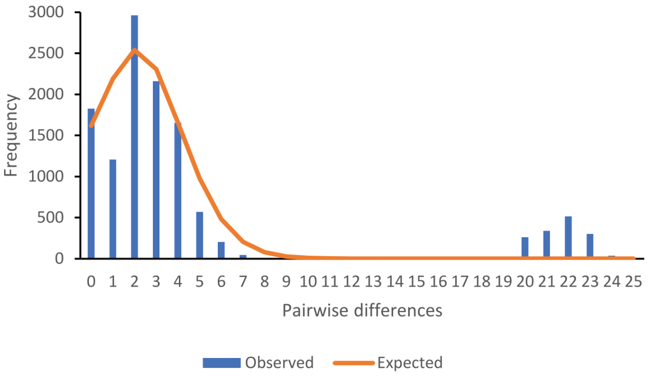
Figure 3. Multidimensional scaling plot (MDS) based on F_{ST} values for *Paracaprella pusilla*. For Cadiz marina (ESCAD) population, four points are represented, each one corresponding to one of the four years when the species was recorded. Populations are coloured according to the region they belong: Northeast Atlantic + Mediterranean (red); South Pacific (green); and presumed native region (blue).

Table 6. Neutrality tests and mismatch distribution analysis for mitochondrial sequences of *Paracaprella pusilla* for each region. Negative and significant values for Tajima's D, Fu's FS and Ramos-Onsis and Rozas' R_2 tests indicate population expansion; SSD = sum of squared deviations between observed and expected distributions; Rg = Harpending's raggedness index; $^*p < 0.05$, $^{**}p < 0.02$.

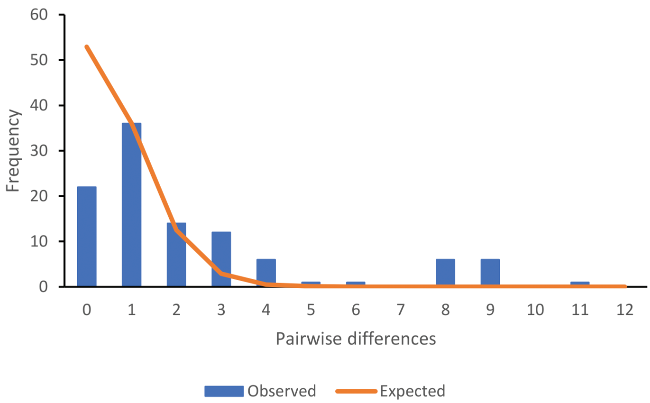
	Northeast Atlantic + Mediterranean	South Pacific	Western Atlantic (presumed native)
Tajima's D	−1.414	−1.543	−1.166
Fu's FS	−1.704	−0.379	−5.661*
R_2	0.047	0.127	0.064
SSD	0.014	0.111*	0.107**
Rg	0.040	0.074	0.034

H4 was the only one present in all monitoring years, and it was the most frequent haplotype found (Suppl. material 4, Table S3). It was only detected in three individuals in 2010, but its frequency increased over time (Suppl. material 4, Table S3; Fig. 2). The remaining haplotypes were only detected in one or two specific years. For instance, haplotypes H1, H2, and H6 (the second the most common haplotype in the spatial study; Table 3; Fig. 2), were found in 2010 and their frequency increased in 2011, but they disappeared afterwards. Haplotype H7 was also present in 2010, not detected the next year, but detected again in 2016 and disappearing again in 2017. Finally, haplotypes H3, H5, H8 and H9 were only detected in one of the years. Overall, the

a) Northeast Atlantic + Mediterranean



b) South Pacific



c) Presumed native region (Western Atlantic)

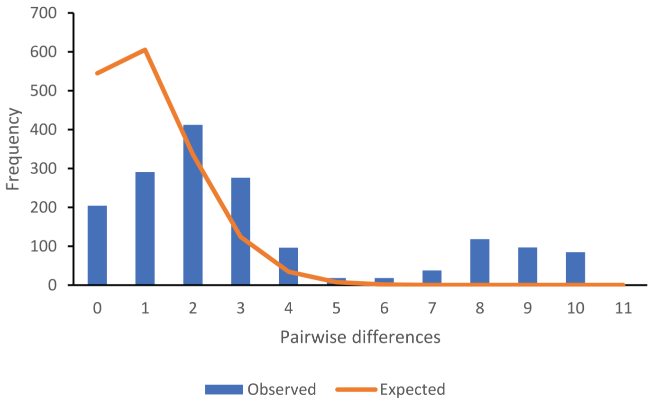


Figure 4. Mismatch distribution of *Paracaprella pusilla* for each region. a) Europe (Northeast Atlantic + Mediterranean), b) Australia (South Pacific), and c) presumed native region. Blue bars show the observed frequency distributions and the orange lines represent the expected ones under the sudden expansion model.

Table 7. Neutrality tests for mitochondrial sequences of Cadiz marina (ESCAD) population over time. Negative and significant values for Tajima's D, Fu's FS and Ramos-Onsis and Rozas' R_2 tests indicate population expansion; $^*p < 0.05$; $^{**}p < 0.02$.

	2010	2011	2016	2017
Tajima's D	0.907	−1.020	0	0.387
Fu's FS	4.096	4.970	4.946	4.221
R_2	0.190	0.079	0.472	0.171

diversities of haplotype (*Hd* = 0.764) and nucleotide (π = 0.00598) were high (Suppl. material 4, Table S3). Haplotype diversity showed a linear pattern of decrease over time (Suppl. material 4, Table S3), with maximal values occurring after the species' initial discovery at Cadiz marina in 2010 (*Hd* = 0.885), and lowest values occurred in the last monitoring year (*Hd* = 0.343). Nucleotide diversity ranged from 0.00169 in 2017 to 0.01515 in 2016 (Suppl. material 4, Table S3).

The F_{ST} statistics showed intermediate levels of divergence between years, with significant values ranging from 0.131 (2010–2011) to 0.261 (2010–2017). Significant differentiation was found between years 2010, 2011 and 2017. Only the year 2016 did not show genetic differences from the other years during the monitoring period, but this could be an artefact due to the low sample size (*N* = 3). In the MDS plot, the year 2017 appeared more separated from the remaining monitoring years carried out in the Cadiz marina population (Fig. 3).

Finally, Tajima's D, Fu's FS and Ramos-Onsis and Rozas' R_2 were negative and not significant for all years (Table 7), which indicated that the Cadiz marina population was not under an expansion phase.

Discussion

Conspecificity of *Paracaprella pusilla* populations

Unlike other caprellid taxa with a wide distribution, such as *Caprella penantis* (Cabezas et al. 2013a) or *C. andreae* (Cabezas et al. 2013b), the absence of population genetic structure (Fig. 1; Suppl. material 2, Fig. S1), the small variation of studied mitochondrial markers, and no differentiation (except one indel in the 28S gene) in nuclear markers reveal that the populations of *Paracaprella pusilla* that we studied did not harbour any cryptic species. Moreover, our analyses confirm that *P. pusilla* and the morphologically close *P. tenuis* are monophyletic and formed highly supported clades (Suppl. material 2, Fig. S1; Suppl. material 5, Fig. S2B). Therefore, our findings support the assumption that anthropogenic dispersal is responsible for the broad geographic distribution of *P. pusilla* and confirms that this is a neocosmopolitan species (see Darling and Carlton 2018).

Native range of *Paracaprella pusilla*

The Atlantic coast of Central and South America has been postulated as the most likely native range for *P. pusilla* (Mayer 1903; McCain 1968; Carlton and Eldredge 2009; Rocha et al. 2013) (Fig. 5a). In our study, the six populations sequenced for this region and including the type locality (Rio de Janeiro) accounted for a higher percentage of private haplotypes (66.7%) than that found for all non-native populations sequenced (56.0%). This could be considered an indicator of long-term residency far exceeding the time-frame of human introductions (Wares 2002). However, the non-significant values of the Harpending's raggedness index (Rg) found for the native region, that may also imply a recent expansion of *P. pusilla* to this region, or the high genetic diversity found in some introduced areas, show the complexity to determine with accuracy the native region of the species through isolated approaches. For example, in an increasingly interconnected world, where maritime traffic continuously connects very distant areas, it is difficult to keep the native region isolated. Secondary introductions from populations introduced in remote areas, and, even more importantly, among sites within the native range, occur, which increase the connectivity and possibly also diversity within particular populations. This is particularly true in fouling species, such as *P. pusilla*, which can be found in both natural and artificial habitats in the native region.

There are, however, several aspects that point to the Atlantic coast of Central and South America as the most likely native area for *P. pusilla*. First, most records of *P. pusilla*, both recent and old, come from this area (Ros and Guerra-García 2012). Second, while most records of *P. pusilla* from putative introduced areas are located in artificial habitats (such as those from India, Europe, Australia, Hawaii, and Pacific Mexico and Panama), in the putative native region *P. pusilla* is also common in natural habitats (Ros et al. 2016b). Third, the biogeographic distribution of species of *Paracaprella* (Fig. 5b) reveals that the Atlantic coast of Central and South America has a high diversity of recorded species, which infers that the centre of diversity for this genus may lie in this area. The other area with a high diversity of recorded *Paracaprella* species is a small region of the Pacific coast of Central America. However, all records of *P. pusilla* from this region are recent and, unlike other records of *Paracaprella* species, are located in artificial habitats (Alarcón-Ortega et al. 2015). For all these reasons, we believe that the Atlantic coast of Central and South America is the most likely native range for *P. pusilla*.

Introduction pattern in temperate European waters

Genetic studies have shown that introduced populations are generally much less diverse than the native ones because of the founder effects and post-introduction demographic bottlenecks (Holland 2000; Rius et al. 2015; Viard et al. 2016). However,

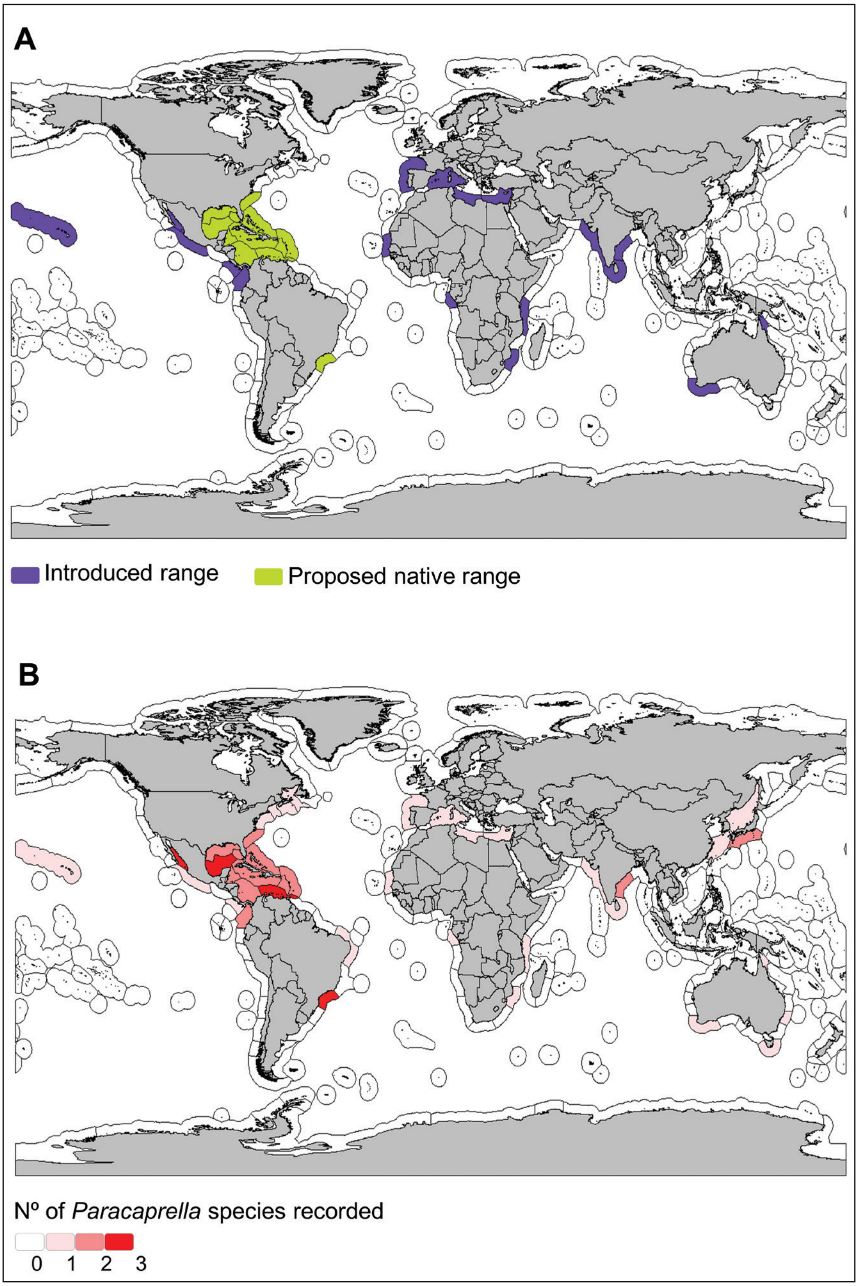


Figure 5. Global distribution of *Paracaprella pusilla* and the genus *Paracaprella* **a** Current worldwide distribution of *P. pusilla* including its introduced range and the proposed native range. Information based on Ros and Guerra-García (2012) and references therein; Ros et al. (2013c); Ros et al. (2014); Ros et al. (2016a); Alarcón-Ortega et al. (2015); Alfaro-Montoya and Ramírez-Alvarado (2018); and Fersi et al. (2018) **b** Number of *Paracaprella* species recorded per marine ecoregions. Information based on Winfield and Ortiz (2013); Mauro and Serejo (2015); Sánchez-Moyano et al. (2015); Lacerda and Masunari (2014). Marine ecoregions follow Spalding et al. (2007).

many introduced populations do not exhibit reduction in genetic diversity and may even exceed native diversity as a result of admixture or high propagule pressure from multiple introductions events (Holland 2000; Roman and Darling 2007; Rius et al. 2015; Viard et al. 2016). The existence of multiple introductions has been widely reported in the marine environment (see Rius et al. 2015), including the two invasive caprellid species which have been genetically studied in Europe (Ashton et al. 2008; Cabezas et al. 2014). In our study, the high genetic diversity found within introduced European populations, similar to that seen in the native ones, coupled with the presence of numerous private haplotypes (Table 3; Fig. 2) suggest that the introduction of *P. pusilla* in temperate European waters likely occurred from multiple introduction pathways and source populations.

Our results support the existence of one of the two main introduction pathways previously suggested by Ros and Guerra-García (2012) and Ros et al. (2013b, c), that is, through the Strait of Gibraltar, from native populations of the Atlantic coast of America. Shipping routes have existed across the Atlantic for more than 500 years (Carlton 1989). Moreover, Europe and the Mediterranean, in particular, are characterized by large clusters of ports with intermediate to high levels of trade (Drake and Lodge 2004; Seebens et al. 2013), and it is estimated that approximately 8% of vessels that travel through the Strait of Gibraltar come from the Caribbean, Gulf of Mexico and Central America (Dobler 2002; Kaluza et al. 2010; Tsiamis et al. 2018). Our mitochondrial dataset suggests that populations from Brazil could be the source of European introduced populations, because their haplotypes grouped closely (Fig. 1) and also because of the lower levels of divergence among them (Table 4; Fig. 3). Moreover, our results indicate that there could exist at least two different introduction pathways through the Strait of Gibraltar: one responsible for the introduction of *P. pusilla* in the Iberian Peninsula (ESCAD, ESSFN, ESBAL) and Israel (ILZIK), and another responsible for the introduction of this species in Tunisia (TNGGB). The presence of haplotype H2, the most common and possibly the ancestral one given its central position in the network (Fig. 1), on both the native Western Atlantic coast and on the non-native European sites (Table 3; Fig. 2), as well as the close relation between haplotypes (Fig. 1) and the low level of genetic divergence among these populations (Table 4; Fig. 3), indicate a clear link between these two regions. According to our results, any of the Brazilian populations, except for Paranaguá Bay (BRPAB), could be the source of *P. pusilla* in European waters, as all of them shared some haplotypes with these introduced populations (Table 3; Fig. 2). In addition, although only three individuals of the Gulf of Gabès (TNGGB) population were sequenced, the exclusive presence of the haplotype H20 in this population but not in the others (where a high number of individuals were sequenced), the absence of haplotype H2 and the lack of any shared haplotypes (Table 3; Fig. 2), as well as the genetic divergence found among this and the European introduced populations (Table 4; Fig. 3) indicate that an independent introduction through the Strait of Gibraltar could have happened. Haplotype H20 only occurred in the native population of Paranaguá marina (BRPAR). This particular Brazilian area might, thus, have been the source for *P. pusilla* in the Gulf of Gabès.

On the other hand, although *P. pusilla* has not been reported in the Suez Canal since Schellenberg (1928) nor in the Red Sea (Zeina and Guerra-García 2016), its recent record in the Israeli coast (Ros et al. 2016a) and the fact that is one of the most abundant caprellid species along the coast of India (Guerra-García et al. 2010), lead us to think of the possibility of these locations as potential sources. Therefore, some individuals of *P. pusilla* could have been introduced to the Mediterranean region from the Indo-Pacific through the Suez Canal (Tsiamis et al. 2018). Future studies including samples from the Indo-Pacific region are necessary.

Paracaprella pusilla was reported for the first time in European waters in the fouling community of a marina on the Atlantic coast of southwest Spain (Ros and Guerra-García 2012) (Cadiz marina, ESCAD, in the present study), and only one year later (2011) the species was found for the first time in the western Mediterranean (ESBAL in the present study) (Ros et al. 2013c). So, according to historical records, the Cadiz marina population could represent the first step in the introduction pathways of this species in this region. However, our molecular results are not in general agreement with this hypothesis. The Palma marina (ESBAL) population had the greatest genetic diversity together with the greatest number of private haplotypes in the introduced range (Table 3; Fig. 2), which indicates that it, and not the Cadiz marina, could be the initial entry point of *P. pusilla* in European waters, and, thus, the source population for subsequent range expansion of this species in this region. Palma marina, in Balearic islands, is the largest port and an important point for commercial cargos, recreational boating, and commercial fishing; this port is one of the most important cruise destinations in the entire Mediterranean (Minchin et al. 2006) and a potential hot-spot of marine bioinvasions (Drake and Lodge 2004; Ros et al. 2013b). As far as we know, the studies of Ros et al. (2013a, c) were the only ones focused on caprellids associated with fouling communities in marinas and ports of Mallorca. So, it is possible that *P. pusilla* was present in Palma marina before its first record in the Cadiz marina. Our data show that European introduced populations are closely related. They shared haplotype H2 (Fig. 2), and the level of divergence between populations was relatively low (Table 4; Fig. 3), indicating that these populations are most likely stepping stones along the same introduction pathway, which is consistent with the scenario of transport by small vessels (Wasson et al. 2001). The stepping-stone invasion pattern is characteristic of many marine invasions and has been reported for other caprellids, such as *Caprella mutica* (Ashton 2006) and *C. scaura* (Cabezas et al. 2014). Thus, *P. pusilla* could have spread from Palma marina (ESBAL) to Cadiz marina (ESCAD), and from there to San Fernando (ESSFN), where the genetic diversity was less (Table 3). Many small vessels of Mallorca overwinter in marinas in southern Spain (Minchin et al. 2006). This, together with the high use of recreational boats on this island (Balaguer et al. 2011), represent a suitable vector for the secondary spread of *P. pusilla* from one location to another (Ros et al. 2013b, c). Unfortunately, only one individual of the eastern Mediterranean population (ILZIK) could be sequenced, which is insufficient to draw any conclusions about the source population and introduction pathway of *P. pusilla* at this locality.

Interestingly, the presence in Palma marina (ESBAL) population of one haplotype (H19) also found in Australia (AUAUS) (Table 3; Fig. 2) suggests that the same pathway or source population may have been responsible for the introduction of *P. pusilla* at these localities. Australian population did not show significant genetic differences from the native region (except with MXSIS) (Table 4; Fig. 3), and half of the haplotypes detected among sequenced Australian individuals were shared with some of the Brazilian populations (Fig. 2; Table 3). One of these haplotypes was H25, a highly distinct haplotype (Fig. 1) that has a specific insertion, which was otherwise observed only in Brazilian populations. All this indicates that the Atlantic coast of South America, namely Brazilian populations, could be the most likely origin for *P. pusilla* in Australian waters.

Temporal monitoring: loss of genetic diversity over time

Our monitoring of the Cadiz marina (ESCAD) population showed a progressive loss of genetic diversity over time (Suppl. material 4, Table S3; Fig. 2). This is consistent with a temporal instability of *P. pusilla* at this location at the westernmost limit of the geographic range of this species in Europe. This species was found in high densities, including ovigerous females, in September 2010, which somewhat refutes the presence of an initial bottleneck due to founder effects, as reported for other marine invertebrates (Pérez-Portela et al. 2012; Bayha et al. 2015). In fact, the high genetic diversity found in this population, comparable with the intrapopulation diversity observed in the presumed native range, together with the presence of private haplotypes (Table 3), indicates that a high number of colonizers arrived, probably from multiple source populations. After its first detection in September 2010, *P. pusilla* was recorded within the following two months. Then, it was not recorded until it was recorded again in the summer of 2011, associated with the absence and presence, respectively, of its main host, *Eudendrium racemosum* (Ros and Guerra-García 2012). In this year, some haplotypes (H1, H2, H4, and H6) found on the initial discovery of the species, were present with higher frequency, but other haplotypes disappeared (H5 and H7) (Fig. 2). These results could indicate that, even during periods when *P. pusilla* was not observed, some individuals could have persisted but remained undetected, due to low abundances (Carlton 2009), and re-established the population when favourable conditions (higher temperatures in summer and presence of *E. racemosum*) returned. Moreover, the presence of new haplotypes (H3 and H8) (Fig. 2) and the weak but significant differences observed between 2010 and 2011 (Fig. 3) could both indicate the existence of a previous bottleneck that unmasked the presence of these haplotypes or that new introductions from nearby populations also occurred. After 2011, *P. pusilla* was not observed during a period of several years until a few individuals appeared in December 2016. Interestingly, these individuals were detected after the finding of this species in a nearby marina (San Fernando, ESSFN in the present study) three months earlier. The presence of the haplotype H4 and the reappearance of the haplotype H7 in ESCAD population, both present in the ESSFN population (Table 3; Fig. 2), clearly suggest a link between these populations. In 2017, more individuals were found at the Cadiz marina and the presence of a new haplotype, only present in ESSFN population (H9), was observed (Fig. 2). This indicates that the reappearance of *P. pusilla* in the Cadiz marina is likely due to the arrival of new propagules from the ESSFN population that resulted in a successful establishment. The decrease in genetic diversity observed (Suppl. material 4, Table S3) consistent with the increasing dominance of one haplotype (H4) (which was not the commonest in the introduced range), indicates that the Cadiz marina population was re-established by a small number of founding individuals ("founder effect"; see Novak 2007; Pérez-Portela et al. 2012; Bayha et al. 2015; Rius et al. 2015). The founder effect is expected to influence the likelihood of long-time survival of NIS, either by reducing the evolutionary potential for adaptation to novel habitat conditions (Sakai et al. 2001; Willi et al. 2006) which inhibit adaptative potential, or by exposing populations to the negative effects of inbreeding (Sakai et al. 2001; Charlesworth and Willis 2009), and the success of the introduction and invasion can be significantly compromised as a result (Sakai et al. 2001; Novak 2007; Wellband et al. 2017). However, some studies have shown that this is not always true, and that low levels of genetic diversity do not prevent the success and spread of non-indigenous species (Roman and Darling 2007; Pérez-Portela et al. 2012; Bariche et al. 2017). Results from the neutrality tests (Table 7) indicate that the Cadiz marina population was not under an expansion phase. This, together with the absence of *P. pusilla* for five consecutive years, suggest that the long-term establishment and success of this species may be compromised. Rather than founder effect, the instability of *P. pusilla* at its westernmost limit in Europe, could be the result of ecological and environmental factors, one of them being the water temperature, as *P. pusilla* is a tropical species and has been mostly found in summer months in its introduced range (Ros and Guerra-García 2012; Ros et al. 2013a). However, the high recreational boating pressure that occurs in this area could increase genetic diversity over time, increasing the likelihood of local adaptation and therefore allowing the expansion of its invaded range. It would be interesting if further temporal genetic analysis could be addressed, preferentially monthly, in all European introduced populations to determine the current status of genetic diversity in this species, and thus, to fully understand how genetic diversity is influencing its introduction process.

Together with the increase in maritime traffic, climate change directly or indirectly increases the spread of NIS into new areas (Carlton 2000; Molinos et al. 2016; Hulme 2017), some of them establishing populations where they previously could not survive (Carlton 2000; Hellmann et al. 2008; Mellin et al. 2016). The increase in the average surface seawater temperatures of the Mediterranean during the last two decades has affected the distribution and abundance of native and non-native species, leading to an enlarged pool of non-native species that have become established and expanded their distributions (Coll et al. 2010; Ulman et al. 2017). This explains why tropical species, such as *P. pusilla*, are penetrating into temperate ecosystems. In fact, the high number of private haplotypes and the star-shaped haplotype network observed in the present study, seems to be a result of the propagule pressure from the species' range in the tropical regions. The occurrence of *P. pusilla* inside marinas, its association with the fouling communities of ships, its ability to spread locally by rafting on detached fragments of these fouling communities (Ros et al. 2016a), and its fecundity (greater than another caprellid, *Caprella scaura*, introduced in this area; Ros et al. 2013c), suggest possible future introductions to other Mediterranean and adjacent localities.

Conclusions

Our study constitutes the first molecular approach to verify *P. pusilla* as a neocosmopolitan species, which has been introduced in European waters from multiple introduction pathways likely including at least populations from Brazil. Molecular, ecological and biogeographic evidences point to the Atlantic coast of Central and South America as the likely native range of *P. pusilla*. While the species appears to be expanding in the Mediterranean, populations from the westernmost distribution limit in Europe (the Atlantic coast of southern Spain) showed a temporal instability. This may indicate that *P. pusilla* is not fully adapted to the environmental conditions in this area, with a water temperature cooler than in the Mediterranean. Further intensive sampling including both native (especially Caribbean populations) and non-native populations of this species, as well as temporal genetic studies, are still necessary to improve knowledge on the diversity of this species in its native and introduced range, confirm the introduction pattern suggested here, and understand the ecological and evolutionary process involved in the introduction success or failure of this species in European waters.

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Supplementary material 1

Table S1. Individual code, specimen voucher, and GenBank accession numbers of COI, 16S, 28S and ITS sequences amplified

Authors: M. Pilar Cabezas, Macarena Ros, António Múrias dos Santos, Gemma Martínez-Laiz, Raquel Xavier, Lou Montelli, Razy Hoffman, Abir Fersi, Jean Claude Dauvin, José Manuel Guerra-García

Data type: molecular data

Link: https://doi.org/10.3897/neobiota.47.32408.suppl1

Supplementary material 2

Figure S1. Bayesian tree of mitochondrial DNA (COI+16S) haplotypes

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Data type: phylogenetic tree

Explanation note: The tree was rooted with *Caprella danilevskii* and *Caprella liparotensis*. Values at the nodes correspond to ML bootstrap support and Bayesian posterior probabilities, respectively. Numbers inside brackets indicate the corresponding haplotypes.

Link: https://doi.org/10.3897/neobiota.47.32408.suppl2

Supplementary material 3

Table S2. Uncorrected pairwise distances between mtDNA haplotypes

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Data type: molecular data

Explanation note: Percentage of average sequence divergence values (based on uncorrected p distances) between *Paracaprella pusilla*, *Paracaprella tenuis*, and the outgroups *Caprella liparotensis* and *Caprella danilevskii*. Distances equal or above 1% are depicted in bold.

Link: https://doi.org/10.3897/neobiota.47.32408.suppl3

Supplementary material 4

Table S3. Changes in genetic variation in Cadiz marina (ESCAD) population over time

Authors: M. Pilar Cabezas, Macarena Ros, António Múrias dos Santos, Gemma Martínez-Laiz, Raquel Xavier, Lou Montelli, Razy Hoffman, Abir Fersi, Jean Claude Dauvin, José Manuel Guerra-García

Data type: molecular data

Explanation note: Year of collection, total number of individuals (N), individuals belonging to each haplotype (H1–H9), haplotype diversity (*Hd*) and nucleotide diversity (π) per year are shown.

Link: https://doi.org/10.3897/neobiota.47.32408.suppl4

Supplementary material 5

Figure S2. Maximum likelihood tree of nuclear markers

Authors: M. Pilar Cabezas, Macarena Ros, António Múrias dos Santos, Gemma Martínez-Laiz, Raquel Xavier, Lou Montelli, Razy Hoffman, Abir Fersi, Jean Claude Dauvin, José Manuel Guerra-García

Data type: phylogenetic tree

Explanation note: **A** Phylogenetic tree of nuclear 28S rRNA. Unfortunately, this gene could not be amplified in *P. tenuis* species. In *P. pusilla*, only two haplotypes were detected, differing only by the presence of an indel. **B** Phylogenetic tree of nuclear ribosomal internal transcribed spacer (ITS). No variation was observed among *P. pusilla* sequences. Trees were rooted with *Caprella danilevskii* and *Caprella liparotensis*. Values at the nodes correspond to ML bootstrap support and Bayesian posterior probabilities, respectively.

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Comparative feeding behaviour of native and introduced terrestrial snails tracks their ecological impacts

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Abstract

A developing body of theory and empirical evidence suggest that feeding behaviour as measured by the functional response (FR) can assist researchers in assessing the relative potential, ecological impacts and competitive abilities of native and introduced species. Here, we explored the FRs of two land snails that occur in south-western Ontario, one native (*Mesodon thyroidus*) and one non-indigenous (*Cepaea nemoralis*) to Canada. The non-indigenous species appears to have low ecological impact and inferior competitive abilities. Consistent with theory, while both species conformed to Type II functional responses, the native species had a significantly higher attack rate (5.30 vs 0.41, respectively) and slightly lower handling time (0.020 vs 0.023), and hence a higher maximum feeding rate (50.0 vs 43.5). The non-indigenous species exhibited a significantly longer time to contact for a variety of food types, and appeared less discriminating of paper that was offered as a non-food type. The non-indigenous species also ate significantly less food when in mixed species trials with the native snail. These feeding patterns match the known low ecological impact of the introduced snail and are consistent with the view that it is an inferior competitor relative to the native species. However, field experimentation is required to clarify whether the largely microallopatric distributions of the two species in south-western Ontario reflect competitive dominance by the native species or other factors such as habitat preference, feeding preferences or predator avoidance. The relative patterns of feeding behaviour and ecological impact are, however, fully in line with recent functional response theory and application.

Keywords
Alien species; functional response; interspecific competition; non-indigenous species

Introduction

Introduction of non-indigenous species (NIS) is largely a consequence of unintentional and intentional human-mediated mechanisms. Once introduced, some NIS adversely affect native species and alter the communities in which they establish (e.g. Dick et al. 2017a). Elton (1958) proposed that characteristics of a native community could be important in providing resistance by native species against successful establishment of NIS. A large literature subsequently demonstrated that interactions (largely predation and competition) by native species could impair or prevent establishment of NIS (e.g. Levine et al. 2004; Smith-Ramesh et al. 2017), though the opposite may also occur (e.g. Levine et al. 2004; Cobián-Rojas et al. 2018). However, the specific mechanisms and thus the predictability of such species interactions and their outcomes remains poorly studied.

Numerous researchers have explored the role of interspecific competition in invasion ecology and its impacts on native ecosystems (e.g. Paini and Roberts 2005). Cases in which a native species competitively excludes a potential invader are particularly interesting, as they may provide valuable insights into context-specific factors that permit the native species to resist invasion (Paini et al. 2008; Zenni and Nuñez 2013). In many other cases, colonizing species may suppress native ones or limit their distributions (e.g. Petren and Case 1996).

One promising method of studying the possible impacts of NIS and the role of interspecific competition is through the use of the "functional response" (FR; see Dick et al. 2017b). Originally developed to study predator–prey relationships, FRs represent the relationship between resource consumption rate and resource density (Holling 1959). Indeed, competition theory refers to the "functional resource utilization response" of competing plant species (Tilman 1977). Using comparative FRs, Xu et al. (2016b) revealed that the impact of the invasive apple snail *Pomacea canaliculata* in relation to native *Bellamya aeruginosa* and introduced *Planorbius corneus* was predictable from the method. Similarly, comparative FRs were used to highlight the strong ecological impact of the "killer shrimp" *Dikerogammarus villosus* on native *Gammarus pulex* (Dodd et al. 2014). Thus, FRs can be used to assess impact on shared resources and hence relative competitive ability of native species and actual or potential invaders with respect to their resource uptake rates (Dick et al. 2017a). In particular, however, this method can move from understanding to predicting invasive species impacts (Dick et al. 2014).

Cepaea nemoralis is a terrestrial snail introduced to North America from its native Western Europe (Örstan and Cameron 2015). Its ornamental value and colourful appearance are responsible for its intercontinental spread by humans (Whitson 2005). The species occupies a wide variety of habitats and can be found in parks and gardens within cities (Ożgo 2012), but does not appear to have significantly harmful effects once introduced (Cowie et al. 2009; Ożgo and Bogucki 2011). This is the case in Windsor and Essex County, Ontario, Canada, where *C. nemoralis* populations are abundant, particularly in urban and disturbed areas. Despite its commonness in these environments, it is rarely found in largely undisturbed woodlands of the region. It is possible that its absence from undisturbed woodlands is at least partly explained by the presence of the native snail *Mesodon thyroidus*, a similarly-sized species found mainly in woodlands including Kopegaron Woods Conservation Area (KWCA), where it often occurs on or in downed logs or under leaf litter. Preliminary surveys of KWCA confirmed the presence of *C. nemoralis* in the more disturbed forest periphery, but the two species never co-occurred in the interior of the forest.

A recent review indicated a significant role of olfaction in detection and selection of food by many terrestrial gastropods, though its importance varies by species (Kiss 2017). It is not clear whether the FRs of species are affected by olfaction nor whether interactions between native species and NIS could be influenced by it.

In this study, we address multiple aspects of the foraging ecology of these two terrestrial snail species, specifically their functional responses, odour detection capabilities and possible interspecific competition. We hypothesized that native, forest-inhabiting *M. thyroidus* may competitively exclude *C. nemoralis* from this habitat type. Specifically, we hypothesized that *M. thyroidus* would exhibit a greater attack rate, shorter handling times (and thus greater maximum feeding rate), shorter search times during olfactory tests, and greater consumption of limited resources in joint foraging experiments with the introduced snail. These predictions follow comparative FR and feeding theory (Dick et al. 2014). To test our hypotheses, we thus used a functional response (FR) framework to compare resource acquisition parameters (i.e. attack rate and handling time) for both these herbivorous snails. We also conducted odour detection experiments to determine whether olfactory cues were important to either species when locating food. Finally, we conducted joint foraging microcosm experiments to observe the relative competitive ability of both snails when placed in a confined environment with limited resources.

Methods

Native *Mesodon thyroidus* snails were found on wooden logs and leaf litter and hand-picked from the ground in KWCA in Leamington, Ontario, Canada, during July 2016. Non-native *Cepaea nemoralis* snails were collected from various urban areas of downtown Windsor, Ontario. Each species was separately housed in transparent aquarium tanks that were covered with fish net mesh to allow oxygenation while preventing egress of snails. Both tanks were maintained in a light- and temperature-controlled chamber (16:8 light:dark regime at 21 °C). Food for snails consisted mainly of grasses, maple leaves (*Acer* sp.) and dandelion leaves (*Taraxacum officinale*) obtained near the Great Lakes Institute for Environmental Research (GLIER), Windsor, Ontario. Snails were fed *ad libitum* during the acclimation period. Dechlorinated water was added to both tanks daily to maintain humidity.

Functional response experiments

Experimental food consisted of dandelion (*Taraxacum officinale*), which is a non-native species in both habitats occupied by the snail species. Dandelion has been used in previous feeding experiments with gastropods (e.g. Desbuquois and Daguzan 1995; Hanley et al. 2003, 2018). Preliminary feeding trials demonstrated that both snail species consumed dandelion, though Hanley et al. (2018) determined that dandelion seedling contained anti-herbivore phenolics and alkaloids and were only moderately acceptable as food to snails (*Cornu aspersum*) in feeding trials.

Snails were used for functional response (FR) experiments following a 24 h food deprivation period to standardize hunger levels. Each FR trial lasted 24 h as preliminary trials showed negligible food consumption over shorter (4 h) periods. Transparent boxes (7.6 × 11.4 cm) were used as arenas to hold food and snails during experiments. A grid composed of 1.3 cm squares was fixed below the box to form a 54-square base (6 × 9). Experimental dandelion leaves were hole-punched to produce circular pellets of uniform diameter (7 mm) as food for the snails. Pellets were placed in the centre of each square to standardize distance between adjacent food items. Original pellets (n = 2) were placed at the centre of the box along the short axis, and subsequent food levels (4, 8, 12, 16, 20, 24, 28, 32, 42, 54) were achieved by adding symmetrically along this axis (i.e. non-randomly).

To begin the experiment, adult and subadult snails were placed at the centre of the arena. Five trials were conducted at each food level for the native *M. thyroidus* and six for the introduced *C. nemoralis*. The arena was uniformly sprayed with deionized water to provide moisture, and boxes were covered with a lid during the trials. At the end of the test period, dandelion consumption was recorded. An event was recorded as full consumption if at least half a pellet was consumed; partial consumption (<50%) was not recorded. Species' FRs were calculated as described below.

Odour detection experiments

Odour preference experiments were conducted in single-species trials with one randomly selected snail individual each. *Mesodon thyroidus* ranged between 1.27 and 2.87 g, whereas *C. nemoralis* ranged between 0.48 and 3.50 g. Fresh dandelion pellets (formed as above) were subjected to one of four treatments: a) desiccation in an oven at 40 °C for 24 h; b) freezing at 0 °C for 24 h; c) pellets from freshly picked leaves; and d) pellets of the same shape but consisting of white paper as a negative control. Freezing significantly reduces volatility of odour compounds in leaves, while oven-drying may cause these compounds to be preserved (Díaz-Maroto et al. 2002). We recorded pellet consumption (as above) for each pellet density (2, 4, 8, 16) and pellet type. We placed a black barrier in the middle of the arena between the pellets and the snail to obstruct its view of the pellets and thereby limited detection by olfactory cues. Time to first contact of a prey item was recorded for each treatment. Each trial was conducted for four hours and repeated with five snails of each species for all food treatments. Species were tested separately (i.e. non-choice experiments).

Joint foraging experiments

The arenas described above for the FR trials were also used to test for possible competition between native and non-native snails. Trials were conducted with a 16:8 light:dark regime at 21 °C. Food pellets hole-punched from dandelion leaves were individually placed in separate squares of the arena (densities 2, 4, 8, 16, 32, 54). Pellets were placed at the centre of the arena and added symmetrically along the short axis of the arena (i.e. successively out to the arena wall as food density increased). For each pellet density tested, five individuals from each species were starved 24 h prior to the trials. We then placed individual native and non-native snails at opposite corners of the shorter edge of the arena facing the pellets. During the 4 h observation, consumed pellets were not replaced, and the number of pellets consumed (defined above) by each snail was recorded.

Data analysis

Statistical analyses were performed in R-3.5.0 (R Core Team 2018). To analyze and model comparative functional responses, we used the FRAIR-0.5.100 package (Pritchard 2017). Rogers' (1972) Type II equation was used to describe the functional response of both species as food resources were not replaced as they were consumed:

$$N_e = N_o(1 - \exp(a(N_e h - T)))$$

where N_e is the number of food pellets consumed, N_o is the initial number of food pellets, a is attack rate, h is handling time, and T is experimental duration (which was set at 1 in the present study as we wished to compare FR parameters for both species over the same period of time). Maximum feeding rate was thus calculated as 1/h. Models were bootstrapped (n = 2000) to generate 95% confidence intervals for each species' functional response curve. Species differences in attack rate (a), handling time (h) and maximum feeding rate ($1/h$) were analyzed using frair_compare() option within the FRAIR-0.5.100 package. Here, as the time for feeding was the same for both species and set as 1 above, a and h were used as unitless, comparative metrics consistent with many previous studies (e.g. Paterson et al. 2015; Anderson 2016; Pritchard et al. 2017), though other researchers have applied units (e.g. Rall et al. 2012, Lefébure et al. 2014, Li et al. 2018). In the latter case, attack rate (a) refers to the volume or area searched per unit time by a consumer, whereas handling time (h) refers to the time spent per unit of resource in activities such as capturing, subduing, killing, ingesting and digesting that resource unit (Barrios-O'Neill et al. 2016; Li et al. 2018).

To compare differential responses to food treatments and delineate interactions of independent variables in the odour detection experiments, we conducted an ANCOVA analysis with factors Species and Food Treatment and continuous variable Food Density, and their interactions. From 160 total observations, 52 instances in which individuals made no contact with the food (regardless of treatment type) were omitted. Nine other instances were also removed from the analysis: four cases in which technical/equipment difficulties caused delays in recording time to pellet contact, four in which snails partially consumed the barrier intended to limit detection to olfactory cues, and one where the barrier became damaged from repeated use and was unable to fully hide the pellets. Detection times were $\text{Log}_{10}(x+1)$-transformed prior to analysis.

Results from joint foraging experiments were analyzed with a paired *t*-test by examining pellet consumption by each snail species across each of the six resource level classes. Each food class was represented five times.

Results

Both snail species conformed to a Type II functional response, though *C. nemoralis* has not reached the curve's asymptote and *M. thyroidus* individuals exhibited a significantly greater feeding ability with increasing food levels (Fig. 1). There was no overlap in 95% CIs, indicating substantially higher feeding efficiency and rate for the native compared to the introduced snail (Figure 1). *M. thyroidus* had a significantly greater attack rate ($a = 5.30$) than *C. nemoralis* ($a = 0.41$) ($z = -9.97$, $P < 0.001$), as well as a slightly shorter but non-significantly different handling time ($h = 0.020$ versus 0.023; $z = 0.25$, $P = 0.800$). Corresponding maximum feeding rate was higher for the native species (50.0 vs 43.5 pellets over the experimental time; see Fig. 1, Table 1).

Mean food detection times for native *M. thyroidus* (1585 s, SE = 369 s) across treatments were shorter than for non-indigenous *C. nemoralis* (1970 s, SE = 266 s). $\text{Log}_{10}(x+1)$-transformed detection times for food resources were significantly shorter for *M. thyroidus* than for *C. nemoralis* (ANCOVA, $F_{1,83} = 9.10$, $P < 0.01$). This was the case for all treatments, with the exception of the "paper" treatment, where *M. thyroidus* took longer to detect the pellets on average (3937 s) than *C. nemoralis* (2094 s). Food density was also significant ($F_{1,83} = 7.27$, $P < 0.01$), as average detection times generally decreased with increasing food density for all but one food level ($n = 8$ pellets). Furthermore, food treatment types differed significantly in detection times ($F_{3,83} = 4.02$, $P < 0.05$) (Table 2), with "paper" averaging the longest time to detection (2764 s) and oven-dried foods the shortest (1334 s). Time to first contact was also affected by a species*food treatment interaction ($F_{3,83} = 3.19$, $P < 0.05$) (Fig. 2).

The joint species foraging experiments demonstrated that feeding activity of *M. thyroidus* was significantly higher than that of *C. nemoralis* across a variety of food resource levels (paired *t*-test, $t = 4.2$, df = 29, $P < 0.001$) (Fig. 3).

Table 1. Rogers' Type II Functional Response parameters (± SE) for native (*M. thyroidus*) and non-native (*C. nemoralis*) snails, including attack rate (*a*), handling time (*h*), and maximum feeding rate (1/*h*).

Species	*a*	*h*	Maximum feeding rate (1/*h*)
Mesodon thyroidus	5.30 (0.49)	0.020 (<0.01)	50.0
Cepaea nemoralis	0.41 (0.05)	0.023 (0.01)	43.5

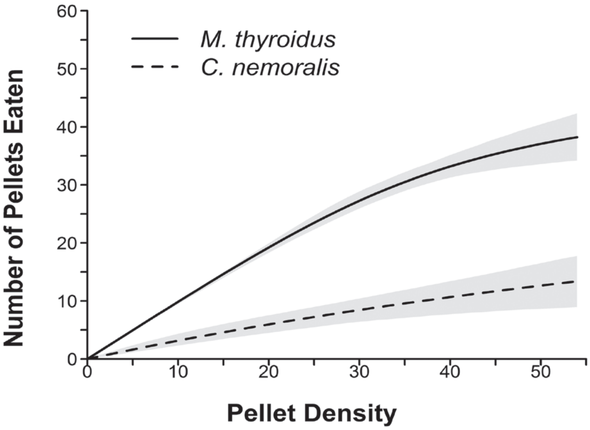

Figure 1. Fitted functional response curves of native *M. thyroidus* (solid line) and introduced *C. nemoralis* (dashed) with 95% CI bands (grey).

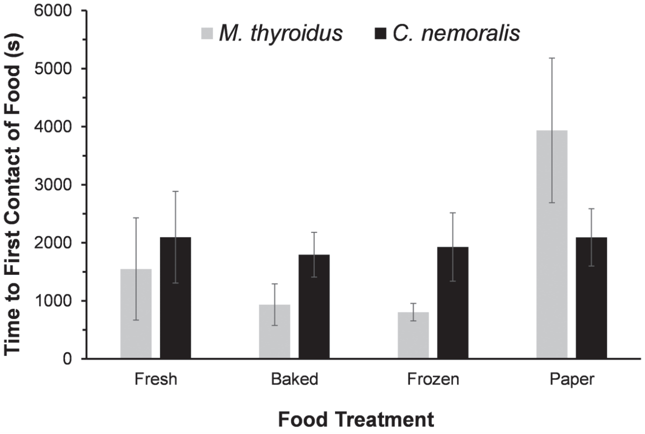

Figure 2. Mean (± SE) food detection times of native *M. thyroidus* (gray) and introduced *C. nemoralis* (black) snails across different food treatments.

Table 2. Results of ANCOVA test assessing effect of Species, Density, and Food Treatment on detection time from the olfaction experiment.

	df	F value	P
Species	1	9.1	0.0034
Density	1	7.3	0.0085
Treatment	3	4.0	0.0100
Species*Density	1	1.7	0.2026
Species*Treatment	3	3.2	0.0280
Density*Treatment	3	1.2	0.3300
Species*Density*Treatment	3	0.2	0.9022
Residuals	83		

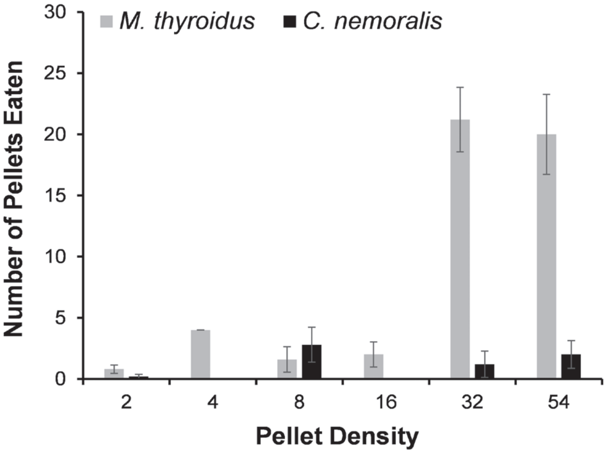

Figure 3. Mean (± SE) pellets eaten in joint foraging experiments across increasing food levels by native *M. thyroidus* (gray) and introduced *C. nemoralis* (black) snails.

Discussion

Application of comparative functional responses has allowed researchers to discriminate between invader species with high and low ecological impact (e.g. Dick et al. 2014, 2017a; Xu et al. 2016b), and may elucidate relative competitive ability (Tilman 1977; Dick et al. 2017b). In most cases examined to date, high functional responses of invaders (relative to their native counterparts) are associated with high ecological impact (Dick et al. 2017a); the opposite pattern is expected with low impact non-indigenous species. Bollache et al. (2008) proposed that the method could be used for NIS likely to invade, thereby allowing forecasts of comparative impact of a putative invader with a complementary native analogue. Further, Dick et al. (2017b) argued that, as with plant competition (see Tilman 1977), FRs of animals may uncover relative interspecific competitive abilities. In our study, we thus examined functional responses of native *M. thyroidus* and introduced *C. nemoralis* snails that occur in different habitats in south-western Ontario. In line with theory, we observed higher FRs for the native species, a consequence mainly of its higher attack rate and maximum feeding rate. The native snail also had a shorter time to first contact across different food densities. The native snail did, however, have a longer time to contact with non-food (i.e. paper pellets), suggesting it is more discriminating than the introduced snail. Indeed, the native species exhibited much shorter times to contact with actual food than with paper, whereas no such variation was apparent with the introduced species (Fig. 2). These experimental outcomes are consistent with the introduced snail having low (or at least unremarkable) ecological impact (see Cowie et al. 2009; Ożgo and Bogucki 2011). This supports general FR theory (Dick et al. 2014), that high FRs are associated with high ecological impact, and vice versa, that low FRs should be associated with low ecological impact. Our data also suggest that the native species is the superior resource competitor, again consistent with FR theory (see Dick et al. 2014, 2017a). In particular, the higher attack rate of the native is congruent with competition theory, as superior competitive ability is likely to be exhibited by the competitor that can best utilise food resources at low food abundance (Tilman 1977), and attack rate quantifies this (see Fig. 1). This also is consistent with the hypothesis that the native species exerts some degree of biotic resistance toward the non-indigenous species.

The two snail species used in our study were collected from separate but nearby habitats. There exist many possible reasons for non-overlapping habitat use by species including interspecific differences in habitat preference and environmental tolerance (e.g. Moreno-Rueda 2007; Książkiewicz et al. 2013), or predation and its avoidance (Morris 2003; Green et al. 2011). It is also possible that non-overlapping distributions could result from intense interspecific competition, with species segregating into different habitats to minimize competition or exploit different resources (Cowie and Jones 1987; Kimura and Chiba 2010). Baur and Baur (1990) demonstrated that land snails competed via both exploitative and interference competition, while Parent and Crespi (2009) proposed that interspecific competition constrained phenotypic variation in Galapagos land snails. However, Chiba and Cowie (2016) found only limited support for exploitation or interference competition among land snail species. Experimental field work is required to assess the respective roles of habitat preference or biological interactions in the microallopatric distributions of these two snail species in south-western Ontario. In addition, molecular analyses of gut contents may improve our understanding of overlap in resource use by these and other species (Waterhouse et al. 2014).

Snail feeding behaviour has been well studied in both terrestrial and marine environments. Much of the recent focus on feeding pertains to mechanisms of food detection, particularly olfaction (e.g. Dahirel et al. 2015; Kiss 2017; Cordoba et al. 2018). To date, only a limited number of studies have addressed functional responses of land snails (see Broekhuizen et al. 2002; Haubois et al. 2005; Giacoletti et al. 2016; Xu et al. 2016a, 2016b; Pusack et al. 2018). In our laboratory study, both native and introduced species conformed with a Type II functional response, consistent with previous studies (e.g. Xu et al. 2016a, 2016b; Pusack et al. 2018). Type II curves are important from the context of population regulation of the resource, as relative risk to prey increases as prey density declines, destabilizing the interaction (Dick et al. 2014). Our study highlighted significantly higher feeding rates by the native snail versus the introduced one, consistent with field patterns of low invader impact and low competitive ability. At the other extreme, Xu et al. (2016b) observed that a highly ecologically damaging invasive snail had much higher feeding rates than its native counterpart. Thus, the FR method is able to predict degree of ecological impact and competitive ability, particularly if combined with species abundances, and can be used to both understand current invasions and forecast the outcome of emerging and future invasions (Dick et al. 2014, 2017b).

Our study utilized a categorical system to assess pellet consumption. One limitation of this approach was that feeding could be assessed as complete when it was only partial, or nonexistent even though some herbivory occurred (<50%). In addition, our results were potentially affected by trial duration (1 d). Had the duration of these trials been extended (e.g. 2 d), some of the observations in the latter category may have flipped from "non-consumption" to total consumption. Finally, it is important to recognize that our study was conducted with only one invasive and one native species (the only species available) and that differences obtained only demonstrate species differences. Confirmation that these differences were due to the origin of the species would require tests with additional species. However, our data and case study fit closely with current FR theory and, together with these numerous other cases (see Dick et al. 2017a), show great potential in predicting ecological and competitive impacts from benign to highly damaging.

Moving forward, further studies of the context-dependency of snail species impacts should focus on mapping FRs onto impact under different contexts, such as various temperature and humidity regimes that might be expected with climate change. In addition, as invaders with low FRs may still exert ecological impact due to high abundance (see Dick et al. 2017b), the impact of native and invasive snails needs to be monitored as relative and absolute abundances change.

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Advancing research on alien species and biological invasions

Forewarned is forearmed: harmonized approaches for early detection of potentially invasive pests and pathogens in sentinel plantings

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Abstract

The number of invasive alien pest and pathogen species affecting ecosystem functioning, human health and economies has increased dramatically over the last decades. Discoveries of invasive pests and pathogens previously unknown to science or with unknown host associations yet damaging on novel hosts highlights the necessity of developing novel tools to predict their appearance in hitherto naïve environments. The use of sentinel plant systems is a promising tool to improve the detection of pests and pathogens before introduction and to provide valuable information for the development of preventative measures to minimize economic or environmental impacts. Though sentinel plantings have been established and studied during the last decade, there still remains a great need for guidance on which tools and protocols to put into practice in order to make assessments accurate and reliable. The sampling and diagnostic protocols chosen should enable as much information as possible about potential damaging agents and species identification. Consistency and comparison of results are based on the adoption of common procedures for sampling design and sample processing. In this paper, we suggest harmonized procedures that should be used in sentinel planting surveys for effective sampling and identification of potential pests and pathogens. We also review the benefits and limitations of various diagnostic methods for early detection in sentinel systems, and the feasibility of the results obtained supporting National Plant Protection Organizations in pest and commodity risk analysis.

Keywords

alien invasive pests and pathogens, commodity risk analysis, early warning, sampling techniques, sentinel plants, pest risk analysis, prediction

Introduction

Invasive alien species (IAS) are amongst the leading global threats to biodiversity, economy and human health (Sarukhan et al. 2005; Early et al. 2016). The number of alien species accumulating worldwide shows no signs of saturation (Seebens et al. 2017). Globalization and international trade have largely facilitated the unintentional long-distance movement of alien plant pests and pathogens into regions outside their native distribution ranges (Seebens et al. 2017). Climate change is also causing natural shifts in the geographic ranges of species, enabling species to migrate and establish in new locations and possibly on new hosts (Musolin 2007; Battisti and Larsson 2015). In the last 200 years, the number of alien invasive forest pathogens has increased exponentially (Santini et al. 2013) and the rate of establishment of alien insect species has nearly doubled over the last 30–40 years in Europe alone (Roques et al. 2016). Relatively recent examples of devastating plant pests and pathogens distributed with live plants include the citrus long-horned beetle (*Anoplophora chinensis* Foster), the box tree moth (*Cydalima perspectalis* Walker), box blight (*Calonectria pseudonaviculata* (Crous, J.Z. Groenew. & C.F. Hill) L. Lombard, M.J. Wingf. & Crous), ash dieback (*Hymenoscyphus fraxineus* T. Kowal), sudden oak death and ramorum leaf blight (*Phytophthora ramorum* Werres, De Cock & Man in 't Veld) (Santini et al. 2013; Prospero and Cleary 2017; Kenis et al. 2018).

Global trade of plants for planting is recognised as the principal pathway for accidental introductions of alien invasive forest and agricultural pests and pathogens worldwide (Kenis et al. 2007; Brasier 2008; Liebhold et al. 2012; Santini et al. 2013; Santini et al. 2018). Once an IAS becomes established and widespread, eradication becomes nearly impossible, the resulting impact and societal costs increase substantially, and only mitigation measures are feasible to help minimise the long-term impact to resource assets. Measures aimed at improving the knowledge base for better prevention of potentially harmful organisms to plants before they are traded will help reduce the risk of new invasions.

Most National Plant Protection Organizations (NPPOs) perform inspections and follow diagnostic protocols of plants for planting and commodities e.g., the European and Mediterranean Plant Protection Organization (EPPO) standards based on lists of known organisms described as invasive and harmful elsewhere (Vettraino et al. 2015). However, alien pests and pathogens often enter in new countries on either non- or unknown hosts, on infected but asymptomatic hosts (e.g. as endophytes, latent infections) or on associated commodities (e.g. soil, wood packaging) (Roques et al. 2015; Vettraino et al. 2017). Thus, there is a need for better tools and strategies to improve early detection of potentially harmful species before they are introduced.

In principle, an early warning system is a major element of disaster risk reduction (Wiltshire and Amlang 2006) developed, for example, to prevent loss of life and/or reduce the economic and adverse effects from a potential disaster. The use of sentinel species, i.e. organisms used to provide an advanced warning of a risk or danger to humans, has a long history in various cultures. One of the earliest uses of sentinel species as an early warning system is from the early 20th century when canary birds (*Serinus canaria* L.) were used in coal mines to warn of carbon monoxide hazards for workers. Sentinel plants in early warning systems are used too as indicators of potential risk associated with damage caused by pests or pathogens based on regular inspections of the plants for signs and symptoms of insect attack or disease (Wylie et al. 2008; Paap et al. 2017; Eschen et al. 2018). For example, sentinel plants have been used to provide adequate warning for damage downy mildew on cucurbit crops, and roses planted at the end of vineyard rows can give an early warning for problems with powdery mildew. In addition, some sentinel plants, are used as indicators of air pollutants (Nouchi 2002).

Two main strategies apply to the sentinel planting concept: sentinel plantations and sentinel nurseries (Figs 1, 2). A sentinel plantation ("ex-patria" plantings sensu Eschen et al. 2018) can be defined as a plantation of non-native plants grown in an environment and monitored to identify biotic agents that affect the growth and vitality of those plants (Roques et al. 2015; Vettraino et al. 2015). A sentinel nursery ("in-patria" plantings sensu Eschen et al. 2018) is defined as a site where native traded plants are planted without phytosanitary treatments in their region of production (exporting country) and monitored to identify pests and pathogens which could be spread with the trade of those plants outside of their native range (Vettraino et al. 2017; Kenis et al. 2018). In this paper, we also consider the sentinel arboretum (Fig. 3) (included as "ex-patria" plantings sensu Eschen et al. 2018). Though not specifically designed as an early warning tool to detect potential plant pests or pathogens, arboreta and botanical gardens can offer another opportunity for sentinel research and contribute valuable information about novel pest–host associations (Britton et al. 2010; Tomoshevich et al. 2013). Procedures for sampling and appropriate protocols for detection and identification of pests and pathogens require standardization for all sentinel systems.

The aim of this paper is to provide an overview of the protocols and techniques useful in sentinel plantings with a focus on: 1) the capacity for sentinel systems to provide useful information to NPPOs for pest and commodity risk analyses, 2) the description of the harmonized diagnostic approach in sentinel plantings, its potential and its relation with the PRA and CMA and 3) sampling, diagnostics and the utility of different techniques in increasing our ability to accurately detect and identify new threats.

Sentinel plants supporting National Plant Protection Organizations

The Food and Agriculture Organization of the United Nations (FAO) defines pest as "any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products" (FAO 2016). However, in the literature plant damaging organisms are frequently divided into "pests" (i.e. invertebrates: arthropods, gastropods, nematodes, etc.; in some cases, also vertebrates) and "pathogens" (i.e. fungi, bacteria and other agents causing plant diseases). Despite the harmful connotation implicit in these terms, it is important to note that not all organisms present in sentinel plantations should be considered injurious. But non-harmful organisms can become so when they change host or their natural environment. As sampling methods and identification protocols differ depending on the organism in question, pests and pathogens will be considered separately as two distinct groups in this work.

Pest risk analysis (PRA) is the process of evaluating biological and economic evidence to determine whether an organism is a pest, whether it should be regulated, and the strength of phytosanitary measures to be taken to reduce the risk of introduction (FAO 2018). PRA is increasingly being replaced by commodity risk analysis (CRA), which instead of focusing on an organism considers a particular commodity (e.g. a plant species) (USDA 2012). The sentinel planting approach is well suited to support such risk analyses: sentinel plantations are focused on identifying potential pests and pathogens that should be the target of PRA, and sentinel nurseries allow identification of pests that may be imported on live plant targets of CRA (Eschen et al. 2018). Moreover, sentinel plantings can also provide information on the extent of damage caused by pests and pathogens, and their biology and ecology (Roques et al. 2015; Fries 2017), all of which are important for PRA.

Despite the great amount of data that can be derived from sentinel plantings, there are several issues that the scientific community and plant health regulators need to address in order to best optimize the use of these data:

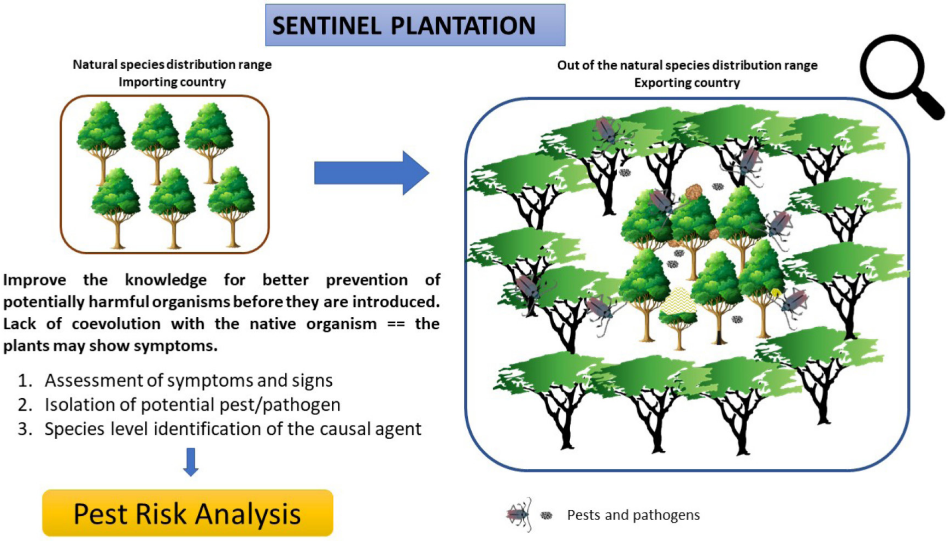
1. There is currently a mismatch between the systems of identification and classification of pests and pathogens used by scientists (e.g. pathogen lineages, molecular OTUs, taxon) and those used by regulators (usually formal species). How data on higher or lower taxonomic levels could be used in plant health regulations or specifically PRA has not been thoroughly examined, although Eschen et al. (2015) suggested that PRAs could target groups of potentially harmful organisms at a higher taxonomic level than species in order to improve plant health protection.
2. The number of unidentified taxa and new pest/pathogen-host relationships in recent sentinel planting studies remains high (Eschen et al. 2018). One main problem is that a PRA is normally only conducted once a pest or pathogen is formally described (FAO 2016). Time limitations and logistical issues restrict the ability of researchers to formally describe unknown taxa in sentinel plantings (Roques et al. 2015; Vettraino et al. 2015; Kirichenko and Kenis 2016). To alleviate this issue taxonomists based in the exporting countries need to be engaged through networking activities.
3. Reliance on DNA methods for detecting a pathogen does not reveal any indication of the viability of that particular organism. Hence, a limitation of high throughput sequencing (HTS) techniques, as suggested by Vannini et al. (2013), is that the risk to plant health remains unproven without a living sample of the pathogen.
4. If numerous potential pests and/or pathogens are detected, the limited resources available for carrying out the labour-intensive PRA process make it necessary to rank potential pests and pathogens according to their perceived risk. Ranking of potential pests that are detected in sentinel plantings need to be based on the biology and abundance of the pest, known substrates or hosts, frequency and severity of symptoms, or damage or known pathogenicity. Expertise or specialist knowledge from different fields (pathology, entomology, forestry) are essential to gain a holistic view.
5. Currently, the sharing of occurrence and disease data from existing sentinel plantings is rare, but a centralized database, as suggested by Britton et al. (2010), needs be used by NPPOs to identify pests and pathogens for PRA. There are ongoing efforts as a part of the International Plant Sentinel Network (http://www.plantsen-

tinel.org) to develop a database to store and share information related to sentinel plantings. This database should be updated with data from regular surveys and have some form of curation. Before data are added to the database, the records should be discussed with the NPPO of the exporting country. In some countries, it is obligatory to notify the NPPO of new findings of pest and pathogens whereas in all cases it is good practice to keep the NPPO duly informed (Eschen 2017). Fostering good relations with the NPPO is vital to enable the establishment and maintenance of the sentinel plantings (Roques et al. 2015). In many cases, NPPOs might also assist in pest/pathogen identification, data provision and further research.

Diagnostic approach in sentinel plantings

Sentinel plantations

In sentinel plantations, non-native plants are grown in a country out of their natural distribution range (e.g. native European trees planted in China) and monitored for potentially damaging agents which may provide useful data for PRA (Fig. 1). If novel pest/pathogen-host plant combinations occur, the plants are likely to develop symptoms due to a lack of coevolution with the native organism (Parker and Gilbert 2004; Vettraino et al. 2015). The assessment of symptoms and signs, along with sampling of symptomatic tissues, and the isolation of potential pest/pathogen organisms, should be prioritized. Therefore, methods and protocols used in sentinel plantations should aim



Improve the knowledge for better prevention of potentially harmful organisms before they are introduced. Lack of coevolution with the native organism == the plants may show symptoms.

1. Assessment of symptoms and signs
2. Isolation of potential pest/pathogen
3. Species level identification of the causal agent

Figure 1. Schematic representation of the sentinel plantation concept. Tree species native to the importing country are planted in the exporting country. Being exposed to the resident pest and pathogens, they should develop visible symptoms.

to characterize damage morphotypes, followed by isolation or collection and species level identification of the causal agent(s) (Roques et al. 2017).

It is necessary to carry out HTS analysis of a representative sample of the propagation material (e.g. seeds) intended to be used before export to the country where the sentinel planting will be located. Knowledge of the plant's endophytic community in its native range can give a baseline for interpretation of, for example, fungi contributing to disease. In sentinel plantation trials in China, absence of controls in the propagation material did not allow confirmation of the Asiatic origin of detected OTUs (Vettraino et al. 2015).

Sentinel nurseries

In a sentinel nursery, native plants are grown in their natural distribution range to identify potential pests or pathogens which could be spread with the international trade of these plants (Fig. 2). In this case, the results obtained will be helpful in CRA (Kenis et al. 2018). Assuming that host-parasite co-evolution of native species might not result in obvious symptom expression, a host shift to a taxonomically similar plant species in the final location of the plant may give rise to novel host-parasite interactions. Therefore, diagnostic methods that can detect endophytic or latent pathogens must be employed (Vettraino et al. 2017) in addition to standardized diagnostics for symptomatic tissue. Thus, sampling must be oriented to both symptomatic and non-symptomatic material. In this system, the use of HTS is useful for screening of the

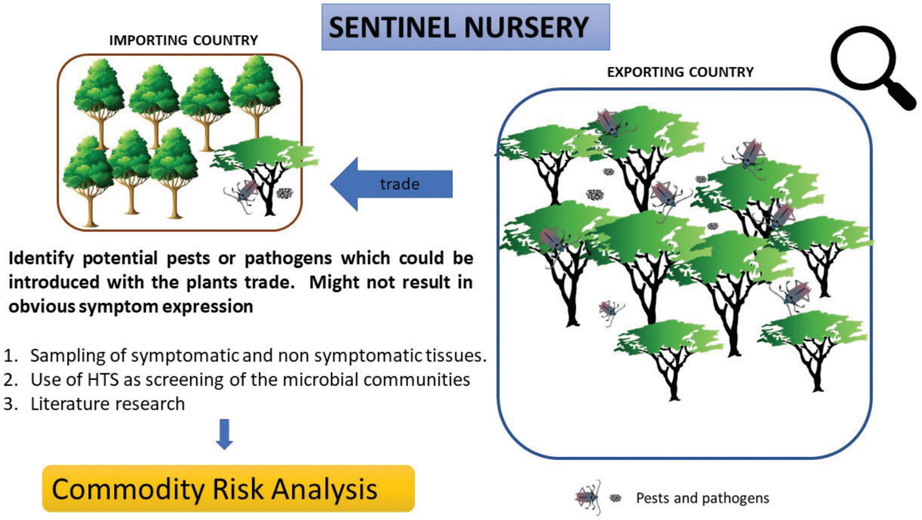


Figure 2. Schematic representation of the sentinel nursery concept. Tree species native to the exporting country and traded with the importing country are regularly inspected for resident pest and pathogens. Because of host-parasite coevolution, visible symptoms may not necessarily develop.

microbial communities even in the absence of symptoms. One possible way to filter large datasets arising from HTS is to group the OTUs according to their functional guild, focusing the sampling and identification on what are grouped as pathogens or opportunistic pathogens. In the case of fungi, online applications, such as FUNGuild (http://www.stbates.org/guilds/app.php), can be used for this purpose as a base for downstream analysis (Nguyen et al. 2016).

Previous fungal studies in sentinel nurseries have not provided conclusive evidence of identified risks but rather provided information that must be analyzed to arrive at a selection of taxa for further study of whether these organisms pose a threat if introduced in a naïve habitat (Vettraino et al. 2017). Information including a collection of isolates, with molecular barcoding and, eventually, taxonomic positions and a database of OTUs resulting from HTS analysis, would greatly strengthen further analyses. Large data sets can be difficult to interpret and require appropriate databases of molecular data and plant pathogens and, certainly, the scientific literature, to make full use of their potential. A limit to data interpretation is the fact that only a small percentage of global microorganism diversity is so far present in the databases. A positive aspect is that a large number of undescribed taxa are present as sequences in molecular databases, which may provide unexpected matches with OTUs from sentinel plantings and useful information on previous detection.

During arthropod studies in sentinel nurseries (Roques et al. 2015), systematic sequencing of the “morphospecies” (defined as a group of individuals that are recognized as probably belonging to a same species based on morphological characteristics) of immature stages and adults was achieved using the “barcode” COI gene to compare potentially, newly recognized species with sequence data already present in global genetic databases. However, only a limited number of the organisms found, essentially lepidopteran larvae, could be identified to the species level. Therefore, arthropod DNA barcoding does not replace the classical approach of morphology-based species identification (Hebert and Gregory 2005; Pires and Marinoni 2010). The combination of both techniques has proven successful in numerous cases (Pires and Marinoni 2010; Okiwelu and Noutcha 2014; Kirichenko et al. 2015) and should be applied also in sentinel nurseries and plantations (Roques et al. 2015).

Sentinel arboretum

A sentinel arboretum (Fig. 3) comprises a broad range of both native and non-native tree species from diverse regions around the world, which can allow testing of various ecological hypotheses on biological invasions, as possible host-shifts, one of the main barriers to establishment of alien plant pests and pathogens, can be examined (Kirichenko et al. 2013; Kirichenko and Kenis 2016; Morales-Rodríguez et al. 2018). Non-native species are exposed to inoculum of native, potentially pathogenic organisms harboured by native trees species growing in the same or nearby environment. An expanded assumption here is that all native and non-native tree species planted in

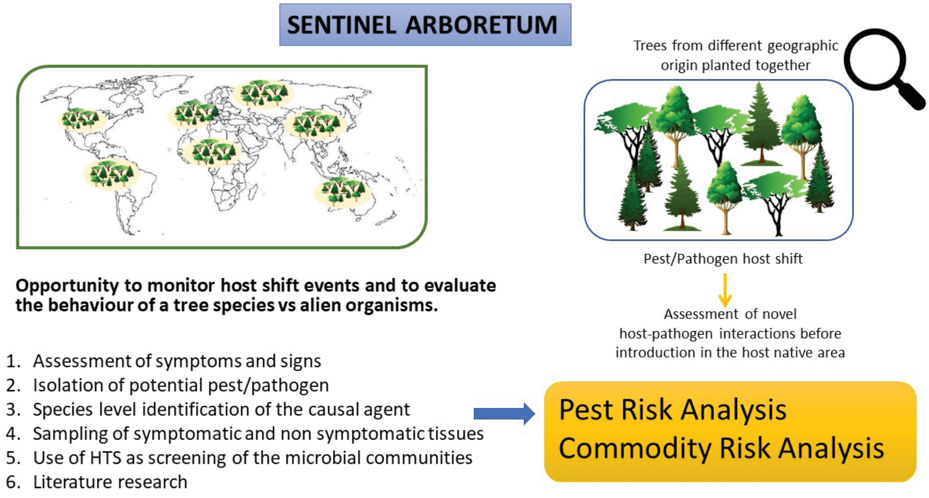


Figure 3. Schematic representation of the sentinel arboretum (botanical garden) concept. The exotic and native tree species cultivated in the same area/environment are cross-exposed to inoculum harbored by each of the species. The identification of causal agents of different symptomatologies provides a list of new pests or pathogens potentially harmful to those plants in their native environments.

the same area are cross-exposed to inoculum harboured by each of the tree species in a latent native-to-native interaction.

Protocols used in sentinel arboreta should aim to characterize damage morphotypes, followed by isolation or collection, and species level identification of the organisms causing these symptoms. The non-native trees might harbour endophytic microflora since the time of their introduction into arboreta as propagation material (e.g. seeds, seedlings, cuttings). HTS can be useful in detecting non-symptomatic native host endophytic species or latent infections, contributing to characterization of the donor host microbiome and to the description of a novel host-shift event. Recently, using HTS and traditional isolation methods, several novel host-interactions between *Quercus* species and fungal pathogens were described in the Ataturk arboretum in Turkey by Morales-Rodríguez et al. (2018). Differing from sentinel plantations, sentinel arboreta may also allow surveys of the recruitment of insects by mature trees, and especially of particular groups, such as xylophagous pests (Roques et al. 2015).

For the three cases of sentinel plantings presented above, confirmation of pathogenicity on the host plant is an essential step for determining the causal agent of disease (Koch's postulates). Thus, collection and isolation of the organism from symptomatic plants is crucial for establishing the causative relationship between a microbe and the disease or symptoms it produces. This procedure, however, is limited to mainly non-biotrophic organisms which can be cultured onto nutrient media. Once the causal agent is known, additional inoculation trials can be designed and carried out to evaluate its potential host range. Colonizing insects observed on sentinel plants must not be incidental, but clearly capable of completing the entire life cycle on the given host, especially when non-native plants are used in sentinel plantings. This process is difficult to ascertain because rearing possibilities on non-native plants could be limited when such plants are only growing within a sentinel plot. One way to distinguish between incidental species and potential pest could be to consider the number of successive colonization events attained over a number of years by an insect species on the same non-native tree. Roques et al. (2015) considered two groups of insects, a first one (38 species) which had shown five colonization events per year, at least on European trees in China, and a second one (7 species) that has been more frequently observed (more than 15 colonization events per year) and probably more capable of switching to European trees. Hence, repeatability and reliability in the observations are critical to drawing sound conclusions on the potential risks to plant health that are needed for PRA and CRA.

Sampling methods used in sentinel plantings

A first step towards the identification of causal agents of damage is usually the observation and recording of symptoms and signs of infections in the field. In the framework of the COST Action FP1401 Global Warning (a global network of nurseries as early warning system against alien tree pests; www.ibles.pl/en/web/cost/globalwarning), an open-access field guide for the identification of damage on woody sentinel plants was published, providing schemes for rough assignment of damage symptoms to relatively broad groups of organisms (Roques et al. 2017).

General considerations for sampling

The successful detection of potentially harmful pests and pathogens in sentinel plantings relies on several conceptual, methodological and organizational factors. Among these, experimental design (i.e. how sentinel plantings are organized, e.g. how many replicates of each tree species), and sampling design (i.e. how, when and what should be sampled) are critical to making sampling as efficient and reliable as possible (Eschen et al. in prep). Similar-looking symptoms might have different causes, and for this reason, the diagnostic procedure can be challenging. Although sentinel plants might be colonized and/or damaged by a broad range of organisms, some general principles about sample collection and preservation apply to all organisms (Kirichenko and Csóka 2017; Prospero et al. 2017). Among these principles, one should consider the following:

1. As different organisms can affect a single plant, the whole plant should be carefully checked for different damage morphotypes (hereinafter referred to as damage characteristic of a certain pest or pathogen) (Tables A1, A2) and the presence of

damaging organisms (Moreira et al. 2017). Samples should be taken from a range of representative symptomatic organs (Nelson and Bushe 2006).

2. Before collecting symptomatic plant material, high-resolution photographs of the whole plant, of the damaged organ(s), and, if present and visible, possible damaging agent(s) should be taken. Categorization of damage morphotypes (Tables A1, A2) might give some hints about the potential causal agents.
3. Cross-contamination from sampling instruments (e.g. secateurs, pruning saw, forceps) should be avoided; this is of particular importance when sampling for pathogens.
4. The best period for sampling varies according to the affected tissues and the suspected causal agents. If possible, at least three samplings per year (spring, summer and fall) should be conducted.
5. Samples should also be taken from apparently healthy tissue to know what healthy plant tissue looks like during normal growth, to potentially detect differences in microbial community composition between healthy and symptomatic tissues, and to study latent infection or endophytes.
6. Proper labelling of sampled material is an essential step without which biological specimens lose their scientific value (Krogmann and Holstein 2010). The minimal data recorded should include locality, GPS coordinates, host plant, date of collection, collector name, and unique identifying number.
7. The stringency of sample disinfection before processing represents an additional variable, especially for biological detection of culturable microorganisms. However, the adoption or not of surface sterilization of samples also represents a conceptual decision. Specifically, in the case of sentinel nurseries, superficial contamination of plants might represent an additional pathway of introduction of alien microorganisms that deserves further attention (Vettraino et al. 2017).

Apart from these general principles, which apply to all groups of damaging agents, there are approaches for sample collection that are specific to the affected plant tissues and causal agent groups (Table A3).

Sampling for detection of pathogens

Pathogens can affect all plant tissues and cause a broad range of symptoms, which could affect the whole plant (e.g. general dieback) or be more localized (e.g. wilting of individual branches). Based on the tissue affected and the type of damage induced (i.e. damage morphotype, Table A1), it may be possible to recognize which group(s) of causal agent(s) is(are) involved. The strategy for sampling symptomatic material varies according to which tissue is damaged (Table A1). It is important to collect not only the symptomatic parts, to optimize the chances of isolating and identify the causal agent(s). To optimize the chances of isolating the causal agent of the symptoms and not a secondary pathogen, samples should include the region where healthy tissue borders infected tissue (Prospero et al. 2017). Evidence of insect attack (holes in the bark, galleries under the bark, sawdust, resin flows) may also be helpful for detecting the presence of pathogens, as insects can act as vectors of other damaging organisms (Weintraub 2007; Zhao et al. 2007; Akbulut and Stamps 2012; Drenkhan et al. 2017).

Sampling for detection invertebrates

Similar to pathogens, sampling of invertebrates varies depending on the affected plant tissue (Table A2) (Kirichenko and Csóka 2017). Invertebrate pests are generally sampled while feeding on plant tissue (to exclude collecting occasional agents that might be on the plant by chance) and preserved for identification. When sampled as immature stages, some arthropods, particularly insects, can be reared to adults in the laboratory as it is the preferred stage for species diagnostics (Gillott 2005). Additionally, plant material with typical arthropod damage can be collected and stored in herbarium collections and used for defining feeding guilds that have added value for identification (Roques et al. 2017). To collect pests, various tools might be used, including nets, umbrellas, collecting trays, aspirators, beating sheets, hand lenses, forceps, and sticky and pheromone traps (Gibb et al. 2006).

Diagnostic approaches to species identification

Information on pests and pathogens are needed for pest- and commodity risk analysis including the organism's identification to the species level and its associated hosts. A variety of traditional, inexpensive techniques and advanced molecular methods are available for identification purposes. The key problem, upon detection of a living pest or pathogen is its correct and rapid identification. Molecular tools can satisfy both of these criteria and have, to some extent, the advantage of being automated. These characteristics make molecular diagnostics as complementary methods to classical morphology-based identification (Rao et al. 2006).

Pathogen identification

Classical techniques

Conventional detection of pathogens involves macroscopic and microscopic examination of symptomatic plant material and isolation of the causal agent. Often, specific isolation protocols, based on optimal requirements for types of pathogens are available, potentially increasing isolation success. However, when working with sentinel plants, there is a risk that causal agents are unknown to science. For this reason, sampled material should be analyzed using a variety of isolation methods, different culture media and temperatures.

Once isolated in pure culture, macroscopic traits, including colony shape, texture and color, and microscopic characteristics of vegetative and reproductive structures are useful criteria for characterization and identification of isolates (Beales 2012).

One problem with the identification of pathogens is the impossibility to grow some organisms on artificial/synthetic media. Obligate parasites such as rust fungi, powdery mildews, viruses and mollicutes require a living host to grow and reproduce. For these organisms vegetative and/or reproductive structure characteristics must be observed on specimens directly from the living host using optical microscopy, or electron microscopy for viruses and mollicutes. Apart from the EPPO protocols, many useful taxonomic manuals, such as Ellis and Ellis (1997), Brenner et al. (2005), Braun and Cook (2012) or Ristaino (2012) can be consulted for morphological identification of fungal, oomycete and bacterial organisms.

Serological tests

Commercially designed kits, such as enzyme-linked immunosorbent assays (ELISA) and lateral flow devices (LFDs) (Lane et al. 2007) are available for detecting and identifying common and known plant pathogens such as the bacterial pathogens *Ralstonia solanacearum* (Smith) Yabuuchi and *R. pseudosolanacearum* Safni (EPPO 2018), and viral pathogens like tomato yellow leaf curl begomovirus and tomato mottlebegomovirus (EPPO 2005). With sentinel systems, species-specific serological tests are however unlikely to prove useful, since many of the target microrganisms could be unknown. Thus, only genus-specific LFDs are useful for rapid in situ screening of samples and the selection of appropriate isolation methods for further laboratory testing. For example, for suspected *Phytophthora* infections, commercial LFDs can give a positive signal enabling the isolation protocol to be oriented towards the use of *Phytophthora* selective media in the laboratory (Lane et al. 2007).

Molecular barcoding

Molecular-based techniques using polymerase chain reaction (PCR) and Loop-mediated isothermal amplification (LAMP) assays are generally more specific and much faster than conventional techniques and can be applied to non-culturable microorganisms. Plant protection organisations routinely rely on diagnostic methods based on PCR assays, e.g. EPPO Standards (https://www.eppo.int/RESOURCES/eppo_standards). The most commonly used markers for molecular identification of fungal pathogens are the ribosomal DNA transcribed spacers, particularly the internal transcribed spacer (ITS) regions ITS1 and ITS2 (Schoch et al. 2012; Romanelli et al. 2014). Although ITS regions perform generally well as barcoding markers for many fungal taxa, this region is less useful for some genera, such as *Fusarium* or *Penicillium*, as these taxa have narrow or no barcode gaps in the ITS regions (Raja et al. 2017). Thus, additional regions must be sequenced. Commonly used regions include the two largest subunits of RNA polymerase II (RPB1, RPB2), β-tubulin regions or translation elongation factor 1 α (TEF1α), which can resolve identification of individual species within the various groups (Schoch et al. 2012). These gene regions are routinely used, depending on the organism (Romanelli et al. 2014). The 16S ribosomal RNA gene and chaperonin-60 (cpn60) are used as bacterial barcode marker genes and to study bacterial phylogeny (Chakraborty et al. 2014). Detection and identification of phytoplasma and spiroplasma are primarily based on 16S rRNA (16Sr) amplification followed by restriction fragment length polymorphism analysis (Bertaccini et al. 2019). When genetic information is available, PCR and reverse transcription PCR are used to detect plant viruses (Jeong et al. 2014).

Rapidly evolving high-throughput sequencing (HTS) technologies enable simultaneous identification of thousands of organism species from numerous and complex samples, with protocols available for viruses, bacteria, fungi, oomycetes and animal pests (Abdelfattah et al. 2018; Tedersoo et al. 2018). The available HTS platforms and details for analysis steps are outlined in Tedersoo et al. (2018). Selecting molecular markers of enough resolution, primers of high affinity to templates, negative and positive control samples and reliable reference sequence databases are the most important factors for HTS-based pest and pathogen identification (Tedersoo et al. 2018). Correct reference data are critical in the precise identification of plant pathogens and, at present, not all publically available databases are sufficiently accurate to enable accurate identification (Jayasiri et al. 2015). Thus, it is crucially important to improve and correct pest and pathogen sequences in publicly databases (Nilsson et al. 2014)

Third-generation sequencing technologies such as PacBio (www.pacificbiosciences.com) and Oxford Nanopore (www.nanoporetech.com) present the possibility to sequence long reads. These technologies have not yet been used in sentinel systems. The benefits arising from amplifying other regions (with sequences longer than ITS1 or ITS2), that could give better identification at the species level, are countered by the absence of adequate reference databases to blast the result obtained. Moreover, these sequencing technologies currently have higher error rates compared with Illumina (Weirather et al. 2017). Despite this problem, it is necessary to emphasize that the new HTS system, such as the MinION device from Oxford Nanopore has great promise as a useful tool in field applications since its portability allows for in situ (on-site) analysis and real-time data generation, thus making the workflow fully versatile.

The use of HTS platforms for biosecurity purposes such as identifying latent or potentially opportunistic pathogens in asymptomatic host tissues requires some consideration of the technological limitations, including the quality of data output (e.g. Illumina MiSeq). While bioinformatics processing can provide useful data output for biodiversity studies (e.g. metacommunity analysis), blast searching of filtered sequence data against custom or public databases generally results in a limited number of identified species, but with many OTUs assigned to higher taxonomic levels. This problem arises due to following reasons: 1) the low power of single-marker short sequences in differentiating taxa, 2) the low taxonomic coverage of databases, and 3) sequencing errors accumulated in the output reads (the sum of amplification and HTS errors). The result is a limited number of OTUs assigned at the species level which may give some value to biodiversity studies but not for biosecurity purposes.

Invertebrate identification

Classical techniques

The observation and evaluation of damage on plants is the first step towards a diagnosis of damaging arthropod and nematode pests. Damage morphotypes can be effectively utilized in sentinel planting surveys as an identifier to assign phytophagous pests to certain feeding guilds, prior to species identification using morphology-based taxonomy (Roques et al. 2017). Classical taxonomy based on morphological characteristics is undoubtedly a powerful tool for arthropod and nematode identification, but some limitations exist, mainly due to the immense diversity and existing gaps in taxonomic knowledge. In most cases, keys are useful only for certain geographic regions and are often based on the identification in the adult stage (Gillot 2005). Furthermore, morphology-based taxonomy may not be helpful for discrimination of closely related species (e.g. sibling or cryptic species) (Bickford et al. 2007). Moreover, disagreements between taxonomists on defining morphological characters, redefining and synonymizing the species may complicate species identification procedures (Okiwelu and Noutcha 2014). Developments in visualizing tools (electron, fluorescent and scanning microscopy) have led to immense improvements in classical taxonomy and continue to contribute to the precision of morphological observations of arthropods and their documentation, which greatly increased the accuracy of species identification (Klaus and Schawaroch 2006; Lee et al. 2009). Some biometric parameters of arthropod body characters could provide added value for distinguishing species (Su et al. 2015). The nematode species can be identified based on the morphological features of the sexual organs of adult male nematodes (Seesao et al. 2016). Knowledge of species biology (life cycle, phenology) and ecology (range, habitat, ecological niche, host plant association) may provide important additional data when identifying taxa (Panizzi and Parra 2012).

The rapid development of computer vision technologies has led to applications in highly promising automatized arthropod identification platforms based on multivariate biometric features of the taxon. This novel approach, based fully on classical taxonomy and computer algorithms, allows species identification procedures to be performed even by non-taxonomists, with a high degree of reliability (Watson et al. 2003; Hassan et al. 2014; Yang et al. 2015; Favret and Sieracki 2016; Wang et al. 2017). Despite being highly attractive, automated species identification suffers from a number of limitations, the most significant being the limited applicability of automated platforms which have for now been created only for a few groups of insects (e.g. individual families of Lepidoptera or Diptera) (Watson et al. 2003; Yang et al. 2015; Favret and Sieracki 2016; Wang et al. 2017), whereas other large groups of important arthropod pests remain far outside the scope of these systems. The process preceding the automated species identification can be tedious, including specimen preparation for scanning and precise positioning for digitizing and recognition by the software. In addition, the computer algorithms may not always be perfect and identification accuracy may not be satisfactory. Despite these and other disadvantages, this developing technology and its possible utilization in mobile devices and other digital instruments in user-friendly mode, would be in high demand for modern forestry and agriculture (Wang et al. 2017) and could also be highly applicable to the identification of potential arthropod pests in sentinel nurseries and plantations.

Molecular barcoding

DNA barcoding is a well-known molecular approach to species identification (Hebert et al. 2003), applicable to any life stage of arthropods, including immature stages (egg, larva, pupa) most often be identified reliably to species level by morphological characteristics (Hebert and Gregory 2005). The method can be highly useful in sentinel plantings, where the pests are usually found in immature stages (Roques et al. 2015).

For arthropods, DNA barcoding uses a short genetic marker – a fragment of mitochondrial DNA (mtDNA) of the cytochrome oxidase I gene (COI; barcoding fragment 658 bp) (Hebert et al. 2003). However, this gene might not always be enough to delineate arthropod sibling species robustly and other molecular methods are required, including nuclear sequencing and/or amplified fragment length polymorphism genotyping (Dasmahapatra et al. 2010; Kirichenko et al. 2015).

As for pathogens, one of the limitations of DNA barcoding is the lack of appropriate reference databases, which would cover all formally described arthropods. To date, comprehensive databases have been accumulated mainly for certain insect taxa (e.g. Lepidoptera and Coleoptera on http://www.boldsystems.org/; Ratmasingham and Hebert 2007), whereas other groups of arthropods remain underrepresented. In the existing databases, inaccuracies may also appear which can lead to misidentification. The quality and accuracy of the sequences stored in the genetic databases might not always be satisfactory, especially considering that any user can access and add sequences (Hebert and Gregory 2005). In a recent survey of insects that colonized a sentinel plantation in China, DNA barcoding enabled to reliably identify only one quarter of sample insect species (Roques et al. 2015)

For nematodes, several genes are targeted for identification such as the mitochondrial cytochrome b locus (mtDNAcytb) (Mattiucci et al. 2003), the gene encoding the mitochondrial cytochrome oxidase 2 (COX2) (Valentini et al. 2006) and the mitochondrial cytochrome oxidase 1 (COXI) (Blouin, 2002), the ribosomal RNA of the small (ssrRNA) and large subunit (lsrRNA) (Hu et al. 2001). Other nuclear genes were also selected such as the internal transcribed spacer 1 (ITS1) of rDNA to identify Strongylidae and Anisakidae (Roeber et al. 2013). NEMBASE (http://www.nematodes.org/nembase4), a publicly available database, provides access to sequences and associated meta-data on parasitic nematode expressed sequence tags (Elsworth et al. 2011). WormBase is an international consortium of biologists and computer scientists dedicated to the research community and providing accurate, current, accessible information concerning the genetics, genomics, and biology of *Caenorhabditis elegans* Maupas and related nematodes (http://www.wormbase.org).

Conclusions

Invasive pests and pathogens are major threats to the health of plants and forests. Key to controlling these invasions are preventative measures that will allow for early detection of potentially damaging organisms preferably before they are introduced to a new region. Sentinel plants can have a fundamental role in this early detection and help predict associated risks to plants in the importing country. The three sentinel plantings described offer different possibilities to provide information useful for PRA (sentinel plantations), for CRA (sentinel nurseries), or for studying host-shift events and novel pest/pathogen interactions (sentinel arboreta).

The protocols and diagnostic approaches to follow will therefore vary amongst these systems. For sentinel plantations, the main focus is on symptoms found on the plants and the identification of the causal agent(s) for which classical identification methods are the key. In contrast, the focus for sentinel nurseries and sentinel arboreta should be on identifying a large number of taxa associated with the host irrespective of whether they are causing damage.

HTS technologies are and will continue to play a pivotal role in the study of biological invasions. In sentinel systems, HTS can help filter information on pest or pathogen taxa so as to focus the sampling efforts and identification only on target species. DNA barcoding and metabarcoding are powerful tools that can give an early warning and confirmation of potential causal agents of damage and can permit the study of the microbial community associated with woody hosts to ascertain the origin and functional role of individuals in different environments. However, reliance on HTS data must be weighed against the accuracy of bioinformatics analysis and depth of the sequence database; and be cognizant on what constitutes a positive or negative result (Martin et al. 2016). Inevitably, the combined use of the different identification techniques – morphology-based, classical and molecular approaches – in sentinel systems may prove beneficial in increasing knowledge of potentially harmful pests and pathogens and potential host shifts if introduced to a new region outside their natural range. The information generated can be highly valuable to plant protection agencies in helping to prioritise organisms for PRA and CRA and contributing to the development of preventative phytosanitary measures, ultimately safeguarding forest and tree resources and their native biodiversity.

The following recommendations can be given to promote the use of data collected through sentinel plantings: 1) better communication between scientists and NPPOs at national and international levels, in particular when potentially damaging pests and pathogens are detected, achieved through increased networking and joint training activities; 2) support from scientists for NPPOs by providing updated pest records and a prioritization strategy of detected organisms; 3) clear communication from NPPOs to scientists about data needs and usage for PRA; and 4) recognition of sentinel plantings as a useful tool by NPPOs, for example through the development of a Standard for Phytosanitary Treatments in sentinel plantings.

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Appendix 1

Table A1. Diagnostic approach for the identification of plant pathogens.

Damage morphotype	Main symptoms and/or signs	Causal agent(s)	Diagnostic approach
Foliage (leaves and needles)			
Discolouration and necrosis	Necrotic spots or patches of different shapes and colours, ring- or net-shaped lines, bands, reduced leaf size; possible presence of reproductive structures on necrotic area	Fungi, oomycetes, mollicutes, viruses, bacteria	1. Isolation from symptomatic tissue 2. Molecular barcoding from cultures 3. Serological test from symptomatic tissue 4. Morphological description of signs (OM[1])
Mould	Soot-like or powdery deposit on the surface; mycelial mats, reproductive structures	Fungi	1. Isolation from symptomatic tissue 2. Molecular barcoding from cultures
Rust	Blisters and/or pustules on the surface (fruiting bodies)	Fungi (biotrophic)	1. Morphological description of signs (OM[1]) 2. Molecular barcoding from symptomatic tissue/signs
Mildew	White powdery mycelium and reproductive structures (including fruiting bodies) on the surface	Fungi (biotrophic), oomycetes	1. Morphological description of signs (OM[1]) 2. Molecular barcoding from symptomatic tissue/signs
Reproductive structures (flower, catkins, cones, fruits, seeds)			
Discolouration and necrosis	Discolorations, necrotic spots; reproductive structures (fruiting bodies)	Fungi, bacteria	1. Morphological description of signs (OM[1]) 2. Molecular barcoding from symptomatic tissue/signs
Rust	Blisters and/or pustules on the surface (fruiting bodies)	Fungi (biotrophic)	1. Morphological description of signs (OM[1]) 2. Molecular barcoding from symptomatic tissue/signs

Damage morphotype	Main symptoms and/or signs	Causal agent(s)	Diagnostic approach
Mould	Soot-like or powdery deposit on the surface; mycelial mats, reproductive structures	Fungi	1. Isolation from the symptomatic tissue 2. Molecular barcoding from cultures
Mildew	White powdery mycelium and reproductive structures (including fruiting bodies) on the surface	Fungi (biotrophic)	1. Morphological description of signs (OM[1]) 2. Molecular barcoding from symptomatic tissue/signs
Fruit rot (mummification)	Entire or partial discolourations, chalky or sponge-like appearance, necrotic spots; fungal mycelium and reproductive structures	Fungi	1. Isolation from symptomatic tissue or signs 2. Molecular barcoding from cultures
Stems, branches and twigs			
Butt and stem rot	Bark lesions, eventually with exudates; fruiting bodies	Fungi, oomycetes, bacteria	1. Isolation from symptomatic tissue or signs 2. Molecular barcoding from cultures
Bark necrosis (canker)	Localised necrotic lesions, swollen or sunken, eventually with exudates; reproductive structures (fruiting bodies)	Fungi, oomycetes, bacteria	1. Isolation from symptomatic tissue or signs 2. Molecular barcoding from cultures
Witches' broom	Concentration of young shoots, which are thicker and shorter than normal ones; reproductive structures (fruiting bodies)	Fungi, bacteria, viruses, mollicutes, hemiparasitic plants	1. Direct symptom observation 2. Isolation from symptomatic tissue 3. Molecular barcoding from cultures or symptomatic tissues (e. g mollicutes)
Epicormic shoots/ fasciation	Sprouts growing from dormant buds, flattened, elongated shoots and flower heads	Fungi, bacteria	1. Direct symptom observation 2. Isolation from symptomatic tissue 3. Molecular barcoding from cultures or symptomatic tissues (e. g mollicutes)
Shoot blight or dieback	Discolorations, wilting or crooking from the tip of the shoots, eventually exudates	Fungi, oomycetes, bacteria, mollicutes	1. Direct symptom observation 2. Isolation from symptomatic tissue 3. Molecular barcoding from cultures or symptomatic tissues (e. g mollicutes)
Roots			
Root rot	Wood decay and eventually staining, root exudates; fruiting bodies	Fungi, oomycetes	1. Isolation from symptomatic tissue or signs 2. Molecular barcoding from cultures

[1] Optical Microscopy

Table A2. Diagnostic approach for the identification of invertebrate plant pests.

Damage morphotype	Main symptoms and/or signs	Causal agent(s)	Diagnostic approach[1]
Foliage (leaves and needles)			
Discolouration	Spots, galleries of different shapes, size and colours, mosaic-like discoloration	Insects (leaf-mining, sucking), mites	1. Collecting damaged leaves for presence of damaging agent
			2. Sampling insects from mines, or on leaf surface; herbarizing leaves with typical damage
			3. Rearing larvae to adults
			4. Morphological identification and/or DNA barcoding (MI & DNA[2])
	Chlorosis, yellowing or browning. External symptoms reflect infestation of wood or roots	Nematodes	See the sections "Stems, branches and twigs" and "Roots"
Lack of surface/ tissue parts	Skeletisation, perforation, holes, cut-outs, rough eating	Insects, snails and slugs	1. Identifying damage type
			2. Sampling feeding larvae and adults directly from leaves or by beating branches.
			3. MI & DNA
Other coating/ covering	Foth, wax, spittle, webbing	Insects, mites	1. Sampling damaging agent by removing the coating or opening the construction (nests)
Construction	Nests		2. MI & DNA
Deformation	Rolling, curling, twisting, reduced size	Insects, mites	1. Collecting damaged leaves for damage type identification
			2. Sampling arthropods by opening the rolls and deformed tissues; herbarizing leaves with typical damage
Outgrowth of plant tissue	Galls		3. MI & DNA
Reproductive structures (flower, catkins, cones, fruits, and seeds)			
Discolouration	Entire or partial (spotted) discolouration, necrotic spots	Insects, mites	1. Sampling mites or insect larvae by opening the affected organ
			2. MI & DNA
Other coating/ covering	Presence of resin flow, white dusting, shield or felt-like covering, etc.	Insects (sap-feeders) or mites	1. Sampling mites, sucking aphids, etc. from the affected organ
			2. MI & DNA
Internal damage: tunnels, holes	Damage invisible at the beginning; later detected as tissue deformation, presence of openings and insect frass on the surface	Insects	1. Sampling larvae/adults from damaged organs/tissue
			2. At early-stage, X-ray seeds for the presence of the damaging agent inside
			3. Rearing larvae in damaged organs to adults
			4. MI & DNA
External injuries	Gnawing, rough eating (lack of tissues parts)	Insects	1. Sampling feeding larvae (nymphs) or adults directly from damaged organs
			2. MI & DNA (any development stage)
Deformation	Distorted or shrivelled organs/tissues (especially flowers, conelets)	Insects, mites	1. Sampling by opening damaged organs/ tissues
Outgrowth of plant tissue or abnormal growth	Swollen organs, gall formations		2. MI & DNA (any development stage)

Damage morphotype	Main symptoms and/or signs	Causal agent(s)	Diagnostic approach[1]
Apparently sound seeds	Apparently sound	Insects	X-raying to reveal presence of larvae
Stems, branches, and twigs			
Coating/ covering	Presence of white dust shield or felt-like covering, etc.	Insects (sap-feeders)	1. Sampling insect from damaged surface
			2. MI & DNA
Internal damage: galleries	Damage invisible at the beginning; later detected through the presence of holes on the bark, insect frass on the surface	Insects	1. Sampling by opening bark with holes or insect frass on the surface
			2. Collecting fragments of bark or wood with typical galleries for damage morphotype identification
			3. MI & DNA
Internal damage: embolism of xylem tissue	Disruption of water transport in the tissues (timber) accompanied by external symptoms: plant stunting, wilting and foliage discoloration	Nematodes	1. Remove bark and inspect sapwood
			2. Collect nematodes
			3. MI & DNA
External injuries	Scars on bark, debarking/bark stripped (girdling or pruning)	Insects	1. Sampling the damaging agent feeding on the bark or by opening swollen plant tissue
Outgrowth of plant tissue	Swollen tissues, gall formations		2. MI & DNA
Roots			
Deformations, root knot or galls, necrosis, atrophy	Thickenings in a variety of shapes, stunting, appearance of necrotic spots, dying-off roots. Accompanied by plant stunting, wilting and foliage discoloration.	Insects, nematodes	1. Sample externally feeding larvae
			2. Collect affected fragments of roots, examine externally and dissect knots and galls to find insect larvae or nematodes (using magnification)
			3. MI & DNA
Injuries (internal and/or external)	Debarking/bark stripped, tunnels, holes and/or frass at root collar	Insects	1. Sampling damaging agent
Coating/ covering	Wax, dust		2. MI & DNA (any development stage)

[1]As a rule, morphological identification of damaging agent is applicable to adult stage solely, whereas for DNA-barcoding any development stage can be used; [2]MI & DNA: Morphological identification and/or DNA barcoding.

Table A3. Sampling methods used in sentinel plantings.

	Sampling pathogens	
Tissue	**Collection**	**Preservation**
Foliage	• Whole leaves/needles should be collected, not only symptomatic parts • If symptoms occur on foliage at different stages all developmental stages should be collected • If symptoms concern whole shoots (e.g. wilting), it is likely that the causal agent has infected the twig/branch and not the foliage, which should also be checked	• Leaves/needles should be collected dry and rapidly processed, avoiding long storage • Leaves with diagnostic damage type should be stored in herbarium collection
Reproductive structures[1]	• Whole reproductive structures should be collected • If symptoms occur on foliage at different stages all developmental stages should be collected	• Apart from cones, seeds and some fruits are better kept dry

Shoots, twigs, branches, stems	• Samples should include the region where healthy tissue borders infected tissue. If symptoms occur on a small branch or sprout, the entire symptomatic section of the branch or shoot should be collected • For vascular diseases and to a lesser extent butt and stem rots, symptoms are often only seen when the bark is removed, and the wood exposed	• Wood tissues should be kept in humid conditions and stored cold (5–8 °C)
Roots	• Carefully remove the soil to expose the main superficial roots. Samples should include the region where healthy tissue borders infected tissue • Since roots are generally infected by soil-borne organisms, soil samples should be collected from the rhizosphere of trees with symptomatic roots	• Roots tissues should be kept in humid conditions and stored cold (5–8 °C)
Visible signs of pathogen damage[2]	• Fruiting bodies and mycelial fans (below the bark) are reliable indicators of pathogen presence and should be sampled either alone or with the substrate on which they grow	• Samples should be stored cold (5–8 °C) and processed rapidly to avoid long storage
Sampling invertebrates		
Tissue	Collection	Preservation
Foliage	• Leaves with typical damage caused by endophagous arthropods (mines and galls), which are often host plant specific, should be preserved as herbarium specimens as they might provide essential information for taxon identification at a later stage	• Preserve arthropods in ethanol, either at 70% for morphological identification or 96% for molecular identification • Slugs and snails can be stored in water in sealed containers • Mites should be preserved in a mixture of ethanol and lactic acid • Plant tissues can be preserved until their processing as described above.
Reproductive structures	• Organs with visible damage symptoms should be collected, with immature individuals present inside • The fruits, cones or seeds can be collected from the ground under a tree or by beating branches over sheets or netting • Seeds can be extracted from fruits or cones and a subset of seeds with no visible signs of damage must be X-rayed to assess the possible presence of larvae inside. Collected seed can also be kept in the laboratory until adult emergence	
Shoots, twigs, branches and stems	• Pests feeding on plant tissues can be sampled directly from the surface or by debarking • Immature insect stages hidden in plant tissues can be sampled together with a healthy plant fragment and reared in the laboratory • For assessing the presence of wood nematodes, wood discs, chips or sawdust should be collected from the sapwood of symptomatic trees, if possible at different stem heights for further diagnostics • Stem sections with dark staining in the sapwood often indicating the presence of blue stain fungi, or signs (holes, galleries) of xylophagous insects should also be sampled	
Roots	• The base of the trunk and the roots should be first inspected for the presence of holes and sawdust (frass) and dissected to find pests • Fine feeder roots showing disease symptoms should also be sampled • Litter and soil around the damaged roots should be inspected • For diagnostics of root-knot nematodes fine roots and soil must first be collected	

[1] i.e. flowers, fruits, catkins, cones and seeds; [2] The term 'visible' means everything observable in the field to the naked eye, or with simple, portable magnifying instruments