

Trade patterns of the tree nursery industry in Europe and changes following findings of citrus longhorn beetle, *Anoplophora chinensis* Forster

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Abstract

The trade in plants for planting is a major pathway for the introduction and further spread of alien plants, pests and diseases. Information about the structure of plant trade networks is not generally available, but it is valuable for better assessing the potential risks associated with the trade in live plants and the development of prevention and mitigation measures and policy. The discovery of two larvae of *Anoplophora chinensis* (citrus longhorn beetle – CLB) in 2009, at a nursery importing *Acer palmatum* from China in one of the major Dutch tree nursery areas, has resulted in the creation of a detailed dataset on the intra-European Union trade in its potential hosts. This study describes European imports of the primary host of *A. chinensis*, *Acer* spp., into the Netherlands (1998–2012) and the effects of the finding in a tree nursery area. In addition, shipments of *Acer* spp. from 138 producers in the nursery area in 2009 were analysed in a one-off analysis of intra-EU trade. The volume of *Acer* spp. imports from Asia was stable early during the studied period, and declined to 5% of the initial imports after a period of interceptions, illustrating the effect of regulations. The number of notifications of *A. chinensis* infestations in imported consignments of

Acer spp. increased sharply in the years up to 2007, then declined as imports also reduced. Although plants were shipped to destinations throughout Europe, each producer shipped plants only to few destinations in few countries. Most of the plants were shipped to nurseries in EU countries. These patterns could make it easier to target these high risk destinations for control measures. The lack of transaction records makes it difficult to trace the destination of plants. More systematic electronic record keeping by traders and growers and the data being collated in a database that can be made available to regulatory authorities, together with further studies of plant trade data using network approaches, would be beneficial for improving trace-back and trace-forward and provide better safeguards for plant health and quality.

Keywords

Citrus longhorn beetle (*Anoplophora chinensis* Forster), International trade, Invasive alien insects, Japanese maple (*Acer palmatum* Thunb.), Plants for planting

Introduction

The international trade in plants for planting is a major pathway for the introduction of alien tree pests and diseases (Levine and D'Antonio 2003, Brasier 2008, Roques 2010, Liebhold et al. 2012). The European Union (EU) imports a large volume and diversity of plants for planting every year, and the value of imported plants for planting has increased 60% over the past fifteen years (inflation corrected data from FAOSTAT; R. Eschen unpublished data). The annual number of new alien tree pests and diseases recorded in Europe increases (Roques 2010, Santini et al. 2013), despite national and international regulations aimed at reducing the introduction of new alien species. Alarming, however, trade movements of plant material into and within the EU are not well documented. The best available data about movements of plant material into the EU come from customs authorities, and these data inform the selection of consignments for phytosanitary inspections. These inspections are primarily intended as checks for compliance with prescribed phytosanitary measures in the country of origin, not at quantifying pest abundance. Phytosanitary inspections of EU plant material are carried out at nurseries during the growing season, but, like in other countries (Li et al. 2014), no routine phytosanitary inspections are carried out on EU internal traded plant material. Since 1992, when controls on the movement of goods within the EU were abolished and the EU became a territory without internal frontiers, it has been difficult to obtain data on internal EU trade. This holds true for the plant and tree nursery trade with its wide network throughout Europe and great number of plant species and varieties. Yet, information on destinations of traded plants and the number of destinations of plants from individual producers is extremely important for understanding and quantifying the potential risks associated with the trade in live plants and the development of mitigation measures and policy (Koch et al. 2014, Shaw and Pautasso 2014).

During the first decade of the twentieth century, Japanese maple (*Acer palmatum* Thunb.) became an increasingly popular plant for both outdoor and indoor gardening. Garden retail companies, but also multinational supermarkets, placed large orders with importing companies. However, from 2008–2010 the tree nursery industry in

Europe was disrupted by an increasing number of interceptions of *Anoplophora chinensis* Forster (Insecta: Coleoptera; Citrus Longhorn Beetle – CLB) on *Acer palmatum* originating from China. The taxonomy of this species remains a little confused, with some taxonomists still referring to *A. malasiaca* as distinct from *A. chinensis*. It is possible that *A. chinensis* interceptions and outbreaks in Europe may be from two closely related species or a species complex (Iwaizumi et al. 2014). CLB is native to East-Asia and interceptions of the species are typically associated with imports of live plants such as *Acer* spp. and bonsai (Haack et al. 2010). In the EU CLB is regulated as a quarantine species, based on the high probability of establishment and potential damage (MacLeod et al. 2002). The larvae bore into the wood of live trees, thereby reducing the quality and value of the wood and causing the death of trees. Infested live trees are difficult to recognise as a result of the cryptic lifestyle of the larvae. An important risk compounding factor is that infested plants can remain undetected for up to three years in the cooler climates of the northern Europe (e.g. Netherlands and the UK). Countries in southern Europe present better conditions and the development time is only 1–2 years (Haack et al. 2010, Van der Gaag et al. 2010). In East Asia, adults also emerge after 1–2 years of larval development, mate and lay eggs on new hosts which can be of a large number of broadleaved genera (Lingafelter and Hoebeke 2002), including common European genera such as *Acer*, *Alnus*, *Betula*, *Populus* and *Pinus*. Dispersal distance is limited, and infestations rarely spread further than 500m in five years (Van der Gaag et al. 2010). CLB has been intercepted in Europe since 1980, both during import inspections and post-entry in various countries (Haack et al. 2010). Haack et al. (2010) reported that only three of the European interceptions (5%) between 1980 and 2008 were made at ports of entry into the EU. The remaining interceptions were made at locations past the port of entry in various EU countries, for example in warehouses or nurseries.

Greater focus on CLB by European authorities following a small outbreak of CLB in the Westland area in the Netherlands in 2007 (NPPO 2008) led to more findings and eradication programmes in the following years, including in Germany, France, Italy, Switzerland and the United Kingdom. In all cases, direct links to earlier imports from China confirmed the high risk of this pathway (Haack et al. 2010). Eradication measures were taken in four European countries after discovery of CLB, but the success has varied and was dependent on the time since establishment, host plant density and climate. The eradication in France and the Netherlands is presumed to have been successful, while the eradication and surveys in Italy and Croatia are on-going.

Another discovery of CLB took place in December 2009 in the Boskoop area in the Netherlands (52°4'N, 4°39'E), one of the most important tree nursery areas in the Netherlands. The greater Boskoop area (Greenport Boskoop; Gemeente Boskoop, undated), which is close to Rotterdam, harbours hundreds of tree nursery companies in an area of ca. 400 km² with an annual turnover of at least Euro 200 million. After the finding, the potential conifer hosts *Pinus* spp. and *Cryptomeria* spp., all woody broadleaved plants, which included hundreds of other known host plants, were removed in a radius of about 100 metres. All plants were individually examined and no signs of CLB

were found. Also, an intensive survey within 600 metres from the infested area did not reveal any signs of the pest. Repeated, intensive monitoring surveys within a radius of 1 km surrounding the finding have not revealed any further signs, indicating that *A. chinensis* had neither spread nor established in the area.

Until this event it was presumed that CLB could not establish in the Netherlands, *inter alia* based on a pest risk analysis (PRA) suggesting that the pest was unlikely to establish in southern England, unless two hot summers occur in sequence (Baker and Eyre 2006; but see Van der Gaag and Scholte 2007). A subsequent PRA (van der Gaag et al. 2008) demonstrated that the import of Japanese maples originating from China into the Netherlands had increased to approximately one million units per year and there was also evidence from the UK and the Netherlands that CLB could extend its development time to 3 years. Based on this PRA, EU emergency measures were taken at the end of 2008, which stipulated that additional phytosanitary measures at the places of production in countries where CLB is known to occur and more detailed and destructive sampling upon import into the EU be implemented (Commission Decision 2008/840/EC: European Commission 2008; amended in European Commission 2010 and updated in European Commission 2012). Costs related to destructive sampling of imported consignments were directly charged to importing companies. In 2010, an EU import ban on *Acer* spp. from China was implemented (Commission Decision 2010/380/EU: European Commission 2010). The import ban was lifted in 2012 and host plants from countries where CLB occurs can only be imported under strict phytosanitary measures including destructive sampling prior to export. Due to the long-term risk of earlier imports and trade from China, the Netherlands was requested by the European Commission in early 2010, to provide details of all deliveries, covering a two year period from mid-2008, from the 2km-radius buffer zone around the initial finding of CLB in Boskoop (“demarcated area”, Food and Veterinary Organisation 2010) with shipments to other EU Member States. This has resulted in a detailed database of trade in live trees in Europe, documenting each transaction, ranging from single trees to large consignments, including long distance trade to the outer reaches of the EU and short-distance trade within Boskoop itself. Following the communication of the transaction information, many other EU Member States investigated these plants but no further findings of CLB were made, apart from one lot of two *Acer* plants which could be directly traced to originate from China.

Using this database, this paper describes the trade in woody plants for planting through the Netherlands with particular focus on CLB and *Acer* spp. Although the data relate to one country only, the Netherlands are the main importing and producing country for live plants in the EU (Dehnen-Schmutz et al. 2010). It must be noted that for woody perennial plants Italy is also an important importing country in the EU. Models have shown that super-connected nodes increase the likelihood for a disease to spread in the system and targeting of such super-connected nodes makes control more effective and efficient (Moslonka-Lefebvre et al. 2011, Pautasso and Jeger

2014). We combine information on imports from non-EU countries into the Netherlands, notifications of pest interceptions and the database that was established in response to the recent CLB outbreaks in the Netherlands. Moreover, we assess the relationship between the number of *Acer* plants from Boskoop and the abundance of potential host plants in the receiving regions. This combination of data enables us to describe the trade in *Acer* spp., an important pathway for the introduction of CLB into Europe, concerning both the import and further movement of the plants within the EU. We discuss how patterns in trade, including the number of destinations to which a producer delivers plants, may affect the risk of spreading pests and diseases and the management of outbreaks.

Methods

Import of plants from third countries

Data about the import of all *Acer* species from third countries into the Netherlands were extracted from the import inspection database of the National Plant Protection Organization of the Netherlands (NPPO) for the years 1998–2012. The database includes details on the numbers of imported plants per genera or even species level, as well as data on consignor and consignee. Although not publicly accessible, this database includes much greater detail as compared to the public databases maintained by the statistical bureau of the European Commission (Eurostat) or the United Nations (UN Comtrade: <http://comtrade.un.org/db/>), because the codes in the latter are too generic to trace individual plant species or genera.

Notifications, pest interceptions, outbreaks

The best available data about the movement of pests into the EU come from pest interceptions during phytosanitary import inspections at the port of entry and findings away from the point of entry. Interceptions of pests regulated in Annex Ia and IIa of Council Directive 2000/29/EC (European Commission 2000) and pests recommended for regulation by the European and Mediterranean Plant Protection Organisation (the EPPO A1 and A2 lists; <http://www.eppo.int/QUARANTINE/quarantine.htm>) are recorded in centralised databases by EU countries. For this study, notifications of interceptions of CLB during the period 2000–2012 were obtained from the EUROPHYT database, which contains notifications of interceptions related to trade. There were twenty interception records in 2008 from Lithuania. It was understood that this interception concerned twenty CLB in one consignment from Japan (Bram de Hoop pers. obs.) and we considered this a single interception. Only interceptions on plants that were recorded as originating from non-EU countries (including Switzerland) were retained.

Intra-European trade

Data about all transactions by producers and traders in the Boskoop area for a two-year period (July 2008–June 2010) were digitised and included the identity (genus and species), number, origin and destination of all shipped plants. Only the transactions for 138 producers in 2009, the year with the largest number of records, were analysed, because of the incompleteness of the records for 2008 and 2010 in the database. In some instances the same plants have been traded within the Boskoop area itself and later to customers outside the Boskoop area. Therefore the total number of plants is lower than recorded in the database, but it is impossible to identify plants that were traded multiple times.

In order to minimize the possibility that individual recipients could be identified and to facilitate working with the large number of recipients, as well as graphical representation of the data, the post codes of the recipients were assigned to the corresponding Nomenclature of Units for Territorial Statistics (NUTS) regions. NUTS regions were established by Eurostat and correspond to administrative regions of EU, acceding EU, candidate EU, as well as European Free Trade Association (EFTA), countries at three hierarchical levels. The lowest level (3) was used in this study as it provides high resolution, although it does not correspond to the same administrative level or average surface area in all countries. For example, NUTS 3 corresponds to “départements” in France, and to the usually much smaller “Kreise” in Germany. Plants of all genera combined were shipped to 800 of the 1,453 NUTS 3 regions, but *Acer* spp. was only shipped to 727 of those regions.

The recipients of *Acer* spp. in thirteen of the countries (Belgium, Bulgaria, the Czech Republic, Estonia, Spain, Finland, Italy, the Netherlands, Norway, Poland, Portugal, Slovenia, the United Kingdom) were classified into seven categories: nurseries, garden centres, landscapers (including garden designers and landscape gardeners), traders, private persons, web shops and other recipients, which included local councils. The identification of the categories has to be taken as an approximation, since some recipients may fall into more than one category. The shipments to these countries represent 85.1% of the *Acer* spp. recorded in the database. The recipients in the remaining countries could not be classified based on the available information.

The relationships between producers and the destinations of shipped *Acer* spp. were described as the average number of destinations to which a producer shipped plants (either a country or NUTS 3 region; generality: Tylianakis et al. 2007) and the realised fraction of possible linkages between all producers and destinations (connectance: Dunne et al. 2002).

The relative abundance of eighteen tree genera, that included the most common European tree species, and two miscellaneous groups with the remaining conifer and broadleaved species were taken from Brus et al. (2012), who modelled their distributions on a 1 km² resolution. Distribution data for trees of genera with potential host plants of CLB (as in European Commission 2008: *Alnus*, *Betula*, *Carpinus*, *Fagus*, *Populus* and the “Miscellaneous broadleaved” genera, which we assumed to include the remaining potential host genera) were assigned to the corresponding NUTS 3 regions. The relationship between the number of *Acer* plants delivered to each NUTS 3 region

and the relative abundance of potential host genera in the same region was calculated using Spearman rank correlation. If more plants were delivered to regions with many potential host trees in the natural environment, this would be interpreted as an indication of the risk associated with the trade in plants for planting. Data on population size and area of each NUTS region were obtained from Eurostat and the relationships between those and the number of *Acer* plants delivered to each NUTS 3 region were calculated using Spearman rank correlation.

The distribution of the *Acer* plants shipped from Boskoop to destinations in Europe was plotted on a cylindrical map using the package “sp” (Pebesma et al. 2012) and the graphical representation of the relationships between producers and destinations of the plants, as well as the description of these relationships were done using the package “bipartite” (Dormann and Gruber 2012), both in R 2.13.0 (R Core Development Team 2011).

Results

Import of plants from third countries

During the period 1998–2012, the total number of *Acer* plants imported from non-EU countries into the Netherlands was greater than 21M, in 1244 consignments (Suppl. material 1). The annual number of imported *Acer* plants was more or less stable until 2005 (ca. 2M), but declined sharply to fewer than 100,000 in 2011 and 2012 (Fig. 1). The pattern for the size of consignments was similar. The type of plants varied between large quantities (tens or hundreds of thousands) of seedlings with a diameter of 1 to 2 cm, ready-for-sale “pseudo-bonsai”, i.e. seedlings rooted in shallow trays for retail sales, and larger older trees of at least 5 cm diameter (Bram de Hoop pers. obs.). The majority of *Acer* plants are exported packed in crates as bare rooted stems with a diameter of approx. 1 to 2 cm. One crate can contain hundreds of individual stems. At the nursery that receives the *Acer* consignment the bare rooted stems are planted in individual pots and maintained in a greenhouse to allow root setting and growth to a plant strong enough for sale. This takes at least several months. A limited number of companies (14) imported *Acer* spp. from third countries in 2011 as opposed to 26 and 27 companies in 2007 and 2005 (Suppl. material 1).

In 1999–2002 the main source of *Acer* imports into the Netherlands was Hungary, which became a member of the EU in May 2004 and trade from Hungary is thereafter considered intra-EU. From 2002 onwards the number of *Acer* plants imported from countries where *A. chinensis* is present, such as South Korea and China, became dominant, with imports from China reaching 95% just before the EU banned imports from China. *Acer* imports from Asia declined sharply after 2007. An increasing percentage of imported *Acer* plants came from New Zealand (81,107 of the total 89,070 imported *Acer* plants in 2012), although the actual number of plants is very small in comparison to total imports in earlier years (>2M in 2004). In 2009 ca. 10% of the *Acer* plants imported from China went through the Boskoop area.

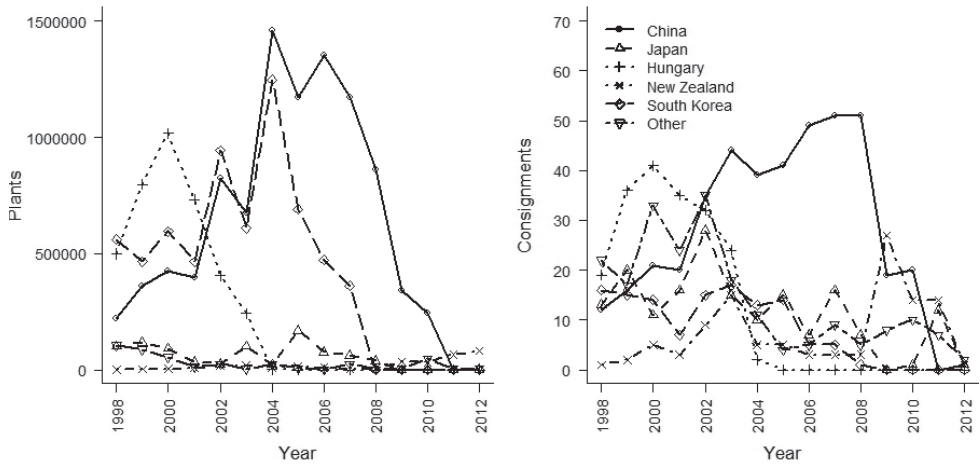


Figure 1. The number of imported *Acer* plants and the number of consignments into the Netherlands by origin. Imports of the remaining 11 countries accounted for a small minority of plants and consignments during this period and are not shown.

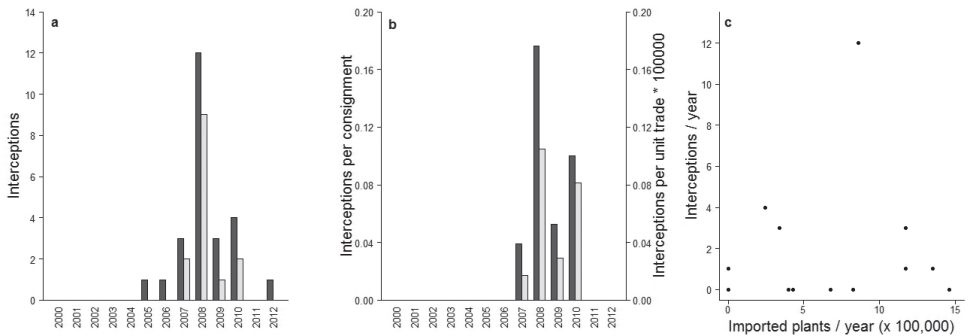


Figure 2. Interceptions of *Anoplophora chinensis* Forster in consignments from non-EU countries in ports of entry into the European Union between 2000 and 2012. a. The total number of *A. chinensis* interceptions of *A. chinensis* in all EU countries combined (black bars) and interceptions of *A. chinensis* from China into the Netherlands only (grey bars). Interceptions after the points of entry, i.e. within the EU, are not included. b. The number of interceptions of *A. chinensis* on *Acer* from China imported into the Netherlands, per consignments and per imported plant (black and grey bars, respectively). c. The relationship between the annual number of imported *Acer* spp. plants from China and interceptions of *A. chinensis* in the Netherlands (2000–2012).

Notifications, pest interceptions, outbreaks

From 2000 to 2012 CLB was intercepted by EU member states on 24 occasions, mainly on imported planting material from China (Fig. 2). The majority of the findings were in *Acer* plants imported from China and a few in bonsai plants from Japan. More than half of the interceptions of CLB were made by the Netherlands. The number of interceptions increased rapidly from 2005 to 2008, but then declined thereafter and no

CLB were intercepted in 2011. No relationship between the number of notifications and trade volume were found ($P > 0.2$). In the course of 2012 two interceptions of *A. chinensis* were recorded on *Acer* plants for planting by Member States of the European Community. One interception concerned a consignment of only two bonsai plants originating from China (Fig. 2). The other interception concerned one *Acer* plant which was intercepted during EU internal movement. The plant had been imported in 2007 from China, prior to enactment of EU emergency measures.

From 2006 to 2010, 190 consignments, containing a total of 3,971,805 *Acer* plants from China arrived in the Netherlands, i.e. ca. 20,900 plants per consignment. In 15 consignments (4.9%) CLB was detected at import inspection and all the plants (269,107) of these consignments were destroyed. One rejected consignment of 44,000 plants was thoroughly investigated by slicing the stems of 16,000 plants to find feeding tunnels and CLB larvae. This method found 9 CLB larvae and another 11 feeding tunnels without larvae, corresponding to an infestation level of 0.056–0.13%.

Intra-EU trade

In 2009, 2,738,974 *Acer* plants were shipped by 138 producers in 34,075 consignments (Suppl. material 2). Typically for Boskoop, only a few companies traded large quantities (7 producers traded more than 250,000 plants per year). The great majority of companies traded relatively small volumes, less than 15,000 plants. Forty-nine producers shipped fewer than 1,000 plants. Over half of the plants from four producers in the Boskoop area were recorded as originating in non-EU countries, and all plants of 104 producers were declared as originating in the Netherlands. The average size of intra-EU consignments is considerably smaller than for import from third countries (318 vs. ca. 18,000 plants) and a great number (19,804) of intra-EU consignments contained 100 or fewer plants.

Thirty-six of the Boskoop producers shipped *Acer* spp. of foreign origin, but only five producers shipped plants of Chinese origin. Of the 23,941 *Acer* plants of Chinese origin (0.87% of the plants traded within the EU), 34.5% remained in the Netherlands, 41.8% went to Germany, 7.1% to Austria and 4.2% to Poland. Small numbers (<1%) were exported to Belgium, Switzerland, the Czech Republic, France, Hungary, Italy and Luxembourg. 68.0% and 31.5% of these plants were shipped by two producers; the remaining 131 plants were shipped by three companies. Two-thirds of the *Acer* plants of Chinese origin were delivered to traders and about one third to nurseries. In 2009 340,696 *Acer* plants were imported from China by traders across the Netherlands; ca. 10% of the Chinese *Acer* plants went through the Boskoop area.

Plants were delivered to 27 countries (Fig. 3). These plants were made up partly from *Acer* arriving in 2009 but also *Acer* imported in previous years and grown on. Three quarters of the *Acer* plants were delivered to destinations within the Netherlands (2,062,404), 8.8% to Germany, 7.1% to the UK and 2.2% to France. It is unknown what proportion of the plants delivered to destinations within the Netherlands in 2009 was exported in subsequent years. The local trade constituted close to a third of the *Acer* plants: 866,028

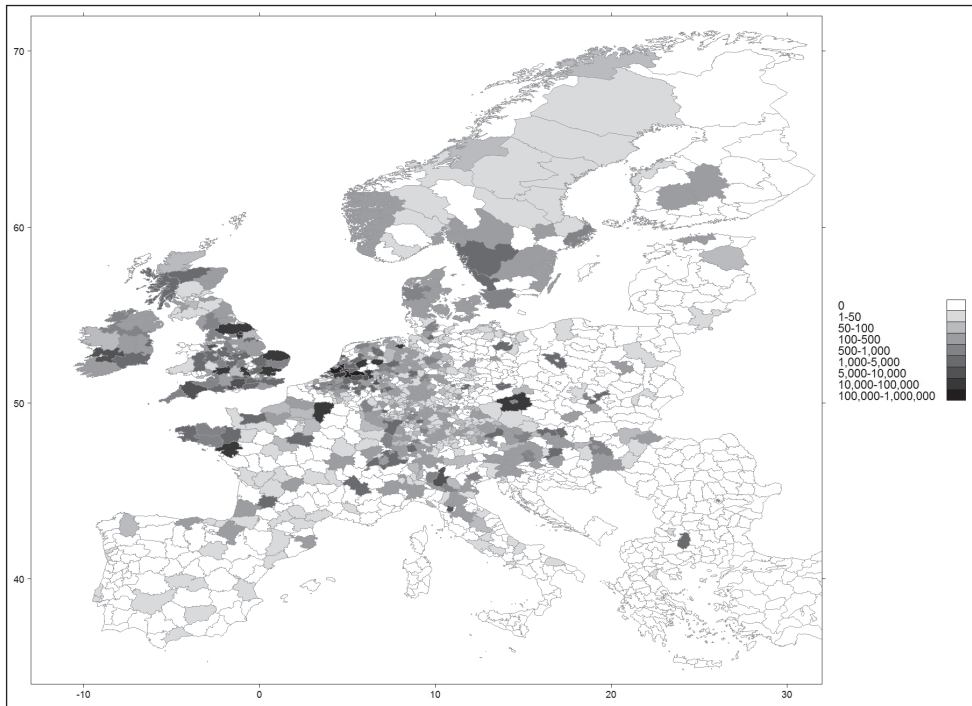


Figure 3. The number of *Acer* plants shipped by 138 producers in the Boskoop demarcated area to NUTS 3 regions throughout Europe in 2009. The darkness of the colouring increases with the number of shipped plants. No data were available for exports to Turkey, because these were not in the database. Map projection is cylindrical.

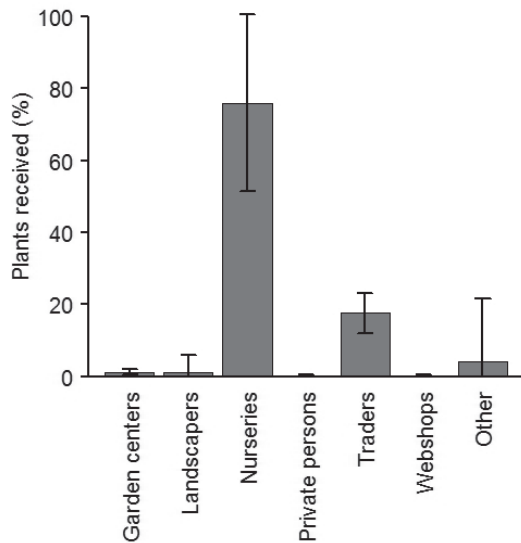


Figure 4. Recipients of *Acer* plants from the Boskoop demarcated area in 2009, as percentage of the total number of shipped plants (2,738,974). Bars indicate average percentages per country and error bars SE.

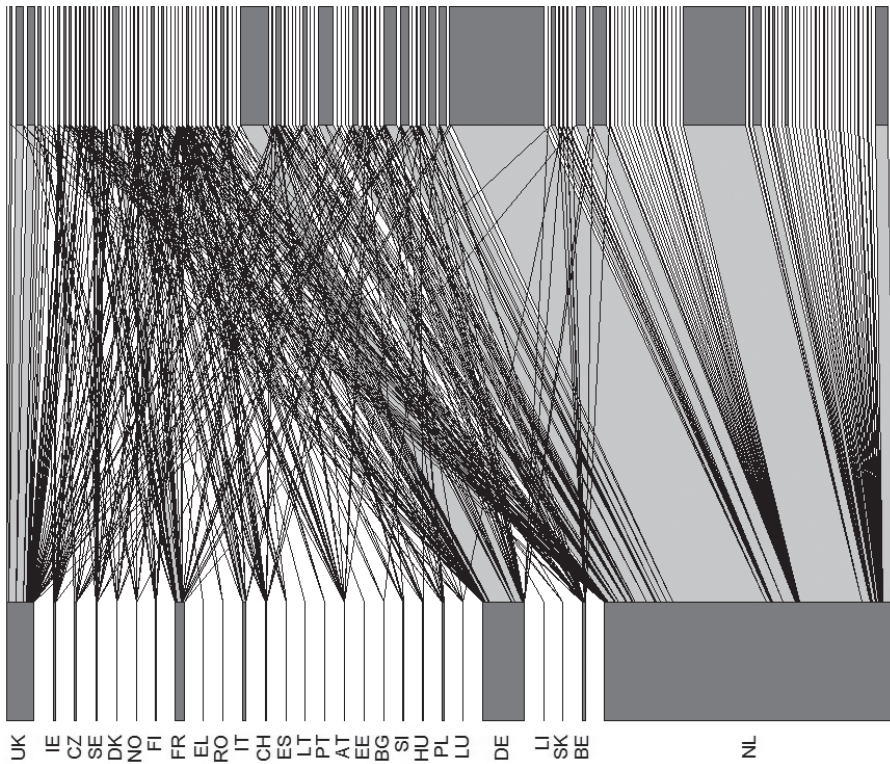


Figure 5. Trade flow of *Acer* plants from individual producers in the Boskoop demarcated area in 2009. Producers are represented by the grey bars on the top of the figure and destination countries by grey bars at the bottom, and the lines between the bars at the top and bottom of the figure indicate the trade in *Acer* plants. The width of the bars and lines indicates the number of plants. The realised fraction of possible linkages between producers and destination countries is 11.3% and 36.7% of the producers only shipped plants to destinations within the Netherlands.

plants were delivered to recipients in Boskoop and Hazerswoude-Dorp in 2,708 consignments; ca. 1,750 to nurseries and 350 to traders (749,402 and 115,291 plants, respectively). Deliveries in Boskoop and Hazerswoude-Dorp involved the majority of the *Acer* producers (101), but it was impossible to identify whether these plants were shipped to a different destination as part of another consignment and some double counting will have taken place. Of the identified destinations in the thirteen countries, an average 75.9% of the plants went to nurseries and 17.6% to traders (Fig. 4). The only exception to this pattern is Bulgaria, where, out of 1,566 plants, 93.5% were delivered to traders. Of the remaining 6.5%, 1.1% went to garden centres and 1.2% landscapers and 4.2% to recipients in the “other” category. Trade to private people and web shops generally involved small numbers of plants per recipient and a very small fraction of the total trade.

Twenty-one producers sold *Acer* spp. only to foreign countries, 66 delivered plants within the Netherlands and also exported and the remaining 51 producers only delivered to destinations in the Netherlands (Fig. 5). The 87 companies that exported *Acer*

spp. each delivered to 1.4 countries on average (generality; Fig. 5). The plants of each exporting producer went to, on average, 5.0 NUTS 3 regions. This number was lower when only exports were taken into account (1.7). Hence, there were large differences between regions and producers (Suppl. material 2). The realised fraction of potential linkages between producers and destination countries (connectance), including the Netherlands, was 11.3% (2% when calculated using NUTS 3 regions). A significant, but very weak, negative correlation between the relative abundance of trees of potential host genera in each NUTS 3 region and the number of *Acer* spp. plants delivered in 2009 was found (Spearman rank correlation $n = 687$, $P = 0.008$, $\rho = -0.100$). In addition, a negative correlation between the number of *Acer* plants delivered and the population size of each NUTS 3 region was found ($P < 0.001$, $\rho = -0.232$).

Discussion

Dutch *Acer* imports from third countries

In order to better understand and manage invasions by tree pests through the trade in plants for planting, analysis of trade networks is urgently needed, but detailed data on this trade are rarely available (Jeger et al. 2007). The data presented here provide a detailed description of the import of *Acer* spp. into the Netherlands and of the intra-European trade in plants for planting. Although import quantities of *Acer* spp. into all of the Netherlands were large at some stage (2 million *Acer* plants imported in 2008–2010), this was small in comparison to trade of all tree nursery products to other parts of the European Union from the Boskoop area alone. Only a few international traders in Boskoop imported tree nursery material from third countries, and in particular China. However, Dutch traders imported relatively large consignments: for *Acer* spp. the average consignment contained 2,000–100,000, up to 384,980 plants. Larger Japanese maple trees were imported in smaller quantities (usually up to 1,000 pieces). However, some of the importers tried to go for optimising profits by selling large quantities to retailers in the Netherlands or other European countries. The description of the imports of *Acer* spp. from third countries reveals the large variability in the origins and quantities imported each year, which was initially driven by the demand for cheap plants. As the interceptions of CLB increased, imports from high-risk origins were reduced by stricter rules on the import of *Acer* plants.

Phytosanitary import inspections are aimed at confirming compliance with prescribed phytosanitary measures in the exporting country, not at quantifying pest abundance. Hence, notifications cannot usually be interpreted as a quantitative measure of pest infestation rate, but in this case it has been possible to directly relate import volume to inspection frequency and infestation rates in Europe, based on data collected through the destructive sampling of thousands of plants in these consignments as stipulated in the EU emergency measures, which is a more reliable detection method than the standard visual inspections. One in twenty imported consignments of *Acer*

from China was infested, which confirms the risk associated with the import of *Acer* spp. from countries where CLB occurs naturally and justifies the emergency measures for this particular pathway. The measures were particularly relevant, due to the low fraction of infested plants in each consignment, as revealed by the destructive sampling. It is unlikely that pests can be found during routine inspections if the levels of infestation are this low. However, the economic cost of the destructive sampling of plants in each of the shipments was considerable. Large bundles of small plants (20–50 pieces of up to 2cm diameter) could be cut with large machines and so the staff cost was limited. However, the economic cost due to loss of plant material was high, approximately Euro 10–50 per plant, depending on plant size. Incursions or early stages of infestation of CLB in Croatia, France, Italy and the Netherlands clearly demonstrate that in some cases it is possible that emerging adults find a mating partner, which could lead to establishment (van der Gaag et al. 2010). This suggests that the high cost of the destructive sampling for detailed inspections is justified. It may, however, be more cost-effective to focus even more on pre-export measures aimed at reducing pest prevalence in consignments.

Intra-EU trade

The vast majority (99%) of the *Acer* plants that were shipped from the Boskoop area in 2009 was declared as originating from the Netherlands, which is risk-free with respect to the potential spread of CLB because the species is not established there. The greatest risk of CLB spread is associated with *Acer* plants originally from China, i.e. less than 1% of the *Acer* plants, but we chose to include all trade in *Acer* plants from the outbreak area, because this provides a better view of the trade in live plants. The plants of Chinese origin were also distributed to destinations throughout Europe (to 14.5% of the NUTS 3 regions). We suspect that some year-to-year variation occurs in the intra-EU trade. However, we were unable to assess and quantify this, because the description of the intra-European trade included only transactions in 2009, restricting our ability to generalise our results, but it is the only year for which we are confident that the data are complete.

A study of the nursery trade for landscapers and retailers in the US indicated that the dominant characteristic of buyer preference was plant quality (Foltz et al. 1993). Other factors which significantly influenced buyer behaviour were offering a diversity of plant species, plant size, origin of the plant, plant price, and the option to pay for purchases with cash. The same study concluded that nursery retailers can best focus on maintaining an existing customer base and hence focus on ensuring a high plant quality. This appears to be reflected in our study by the large number of small trade contacts between companies within Boskoop itself. Such contacts are needed for companies to assemble specific tree packages and satisfy their trade contacts across Europe. Indeed, most Boskoop companies had few relationships with destinations in other parts of Europe. In other words, recipients in other EU countries will normally not obtain plants

from several providers in the Boskoop area, at least not during the period when trade data was assembled. Although *Acer* plants were sent to half of the NUTS 3 regions in one year, the low number of destinations per producer (generality) and the low fraction of potential linkages (connectance) that are realised (Fig. 5) limit the chance that pests and diseases are spread widely by any single producer. This is information that should be taken into account when assessing the risks associated with the trade in live plants, but also when considering risk mitigation options.

The majority of *Acer* plants were sent to destinations in the Netherlands, Germany and the UK and comparatively few plants were sent to southern EU member states, which appears to indicate that there is little demand for *Acer* plants in these countries, or that there are no trade connections. For example, although Italy is a large importer of plants for planting (US\$ 289M in 2009; data from FAOSTAT), the value of plants for planting imported from the Netherlands in 2009 was only a third of the value of imports from France (US\$ 29M imported from the Netherlands). The distribution of the plants to regions within EU countries was uneven, with some regions receiving very large numbers of *Acer* plants from Boskoop and others only few plants. Some studies have used the economic productivity, the length of the road network or the number of airline passengers to explain the number of alien plant pests in a region (e.g. Roques 2010, Paini et al. 2010). In our study, however, there was no obvious link between the number of plants shipped (a proxy for pest approach rate) and the economic output or human population size of a NUTS3 region. Rather, some regions received many plants because of the presence of retailer distribution centres while in other regions, especially in the Netherlands, UK and Italy, there were relatively large or many nurseries. Hence, the distribution pattern of *Acer* plants described here does not necessarily reflect the final planting location of the plants.

Regions with many or large nurseries or retail distribution centres may act as highly connected nodes in the European plants-for-planting trade network, with only few trading steps between the point of import into Europe and the consumer (Shaw and Pautasso 2014) and this small-world network is likely highly effective at distributing pests rapidly throughout the continent. The map in Figure 3 may help to identify the location of such highly connected nodes, but the plants may not stay in those centres for very long and the environment may not be favourable for pest establishment. The onward distribution of the plants from nurseries or distribution centres to retailers and end consumers is likely to lead to a rapid reduction in the number of host plants in a single location, but the data presented here do not allow a description of the further distribution. The dilution of possible infested lots of plants may lower the chance that more than one beetle emerges at the same time and that mating can take place, but it is unlikely that this can avoid establishment of beetles, as indicated by the outbreaks of CLB in Italy. The risk may be higher when members of the public order plants directly from suppliers in East-Asia, for example through the internet. Internet sales were uncommon in comparison with sales through European garden centres or nurseries, although these are increasing (Giltrap et al. 2009, Lenda et al. 2014). The reported findings of individual beetles in private gardens rarely lead to the establishment of

natural populations, but it is not impossible. Examples such as the findings of individual beetles emerging from infested plants originating from imported consignments reported by EU member states (in Germany twice each in 2008 and 2009) highlight the risks associated with this pathway.

It can be argued that consignments that are split up as the plants are distributed throughout Europe are difficult to regulate or control in terms of quality and plant health. High risk locations are nurseries that grow vast amounts of host plants imported from areas where CLB is present, of which there were not many in Boskoop. The imported plants may stay for several months to several years at a nursery for root setting before they are ready for sale. In particular in these high-risk nurseries, more than one beetle may emerge from infested plants, mating may occur and mated females can oviposit on other host plants in or outside the nursery. There are several reports where this has taken place. In France, several beetles were found in a nursery and in Guernsey 10 adults were caught in one greenhouse. At two locations in the Netherlands, infested outdoor trees were found in the neighbourhood of nurseries growing vast amounts of *Acer* plants imported from China for several consecutive years. Also, the outbreaks in Italy are related to nurseries importing and growing *Acer* plants from China or Japan. Hence, recipients of large numbers of plants have an increased risk of receiving infested plants and mating of emerging beetles occurring, which may lead to establishment or further spread of CLB. However, the long development time of *Anoplophora* (2 to 3 years) can desynchronise emergence of beetles at Northern European nurseries, which reduces the risk of establishment and may result in lower growth rates of establishing populations.

Once large consignments are imported, they are either directly marketed via wholesale companies or become increasingly fragmented and it is difficult to trace plants to their true origin. The lack of traceability makes the management of an outbreak more difficult as it takes more time and effort to discover the origin of the plants and the destination of other plants from the same consignment or producer. The experience with data collection following the outbreaks in the Boskoop area revealed that a large number of producers only kept paper records of plant movements, which had to be entered into an electronic database when other Member States demanded that the Netherlands provide these in the aftermath of the Boskoop incident. The absence of readily available transaction records may cause delays in the tracing and management of outbreaks outside tree producing areas. Hence, it is essential that electronic records of transactions are kept, possibly in centralised databases, in order to access such data when required by NPPOs for the purpose of *a priori* risk assessment or tracing of potentially infested plants in the case of an outbreak. Electronic record keeping would also be beneficial for the industry, as rapid eradication and limitation of further spread of pests may mitigate the potential economic and reputational damage of such an event.

Raising awareness and quality levels to reduce the risk of CLB establishment may best be attained by focussing on checks for larger consignments, that is, before they become fragmented (Pautasso et al. 2010). In addition, plant propagation certification systems, as already established for most fruit trees in Europe and elsewhere, may be beneficial for promoting plant health and quality levels of tree nursery products. The

significant but weak negative relationships between the number of delivered plants and the abundance of potential host plants in the natural environment and the population size in the NUTS 3 regions do not provide information that could be used for the assessment of the risk to the natural environment related to the EU-wide shipment of *Acer* spp. *Acer* plants from the Boskoop area are mainly grown as garden plants and it is likely that many of the plants delivered to nurseries for further growth, before sale to consumers or garden centres, would eventually be planted in urban areas. This appears to be in conjunction with the location of the European outbreaks so far, that occurred close to nurseries that traded in *Acer* spp., in particular from China, and not in forests (Roques 2010). It may also be argued that urban trees might be more stressed and more vulnerable to CLB attacks, or that pests are more likely to be detected as a result of the larger number of observers. The higher incidence of CLB in urban areas, or close to nurseries, has positive implications for the chances of eradicating CLB in outbreak areas: the fact that many consignments went to nurseries, most likely for further growth prior to resale, may enable the detection of previously unnoticed CLB infestations, but also poses a greater invasion risk as a result of the increased likelihood that emerging beetles find a mate for reproduction. However, the short dispersal distance of CLB (van der Gaag et al. 2010) may limit spread from the outbreak area, which also facilitates eradication.

Conclusion

It is clear from our data on trade in *Acer* plants from the Boskoop area that live plants are distributed throughout Europe and this illustrates how infested plants could spread CLB far and wide. This is also relevant for the potential spread of other pests and pathogens through the live plant pathway. Despite the detail in the trade dataset presented here, it is impossible to relate the data on intra-EU trade to outbreaks of invasive pests or diseases. This study showed that detailed data on the trade in tree nursery products are valuable for the assessment of risks associated with the movement of large numbers of plants into, and then further through the EU, because the point of entry or production of these products often is not the final destination. Such data may improve the design of spread models and mitigation methods. Moreover, in particular for the benefit of plant health and quality, more systematic electronic record keeping by growers and traders would be supportive for improving trace-back and trace-forward.

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Supplementary material 1

Table S1

Authors: René Eschen, Jean-Claude Grégoire, Geerten M. Hengeveld, Bram M. de Hoop, Ludovic Rigaux, Roel P. J. Potting

Data type: measurement

Explanation note: Summary of dynamics of the import of *Acer* plants into the Netherlands in 1998–2012. The number of importing companies relates to the confirmed importers of *Acer* spp. HU indicates Hungary, AS indicates East-Asia and NZ indicates New Zealand.

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Supplementary material 2

Table S2

Authors: René Eschen, Jean-Claude Grégoire, Geerten M. Hengeveld, Bram M. de Hoop, Ludovic Rigaux, Roel P. J. Potting

Data type: measurement

Explanation note: Summary of the intra-European trade in *Acer* plants from 138 producers in the Boskoop demarcated area in 2009, broken down by country.

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Effects of UV-B radiation on germination characteristics in invasive plants in New Zealand

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Abstract

UV-B radiation represents a potentially selective, yet little studied environmental factor for plant invasions, especially with respect to germination characteristics and seedling establishment in areas of high UV-B exposure such as New Zealand. To explain invasive potential of plant species pre-adaptation and local adaptation to selection factors in the invaded range are two frequently consulted concepts. To test for the relevance of these mechanisms, it is necessary to compare both invasive and non-invasive species, as well as native and exotic origins of invasive species. In the present study, germination success of two congeneric species pairs of the genera *Verbascum* (Scrophulariaceae) and *Echium* (Boraginaceae) were investigated under high UV-B intensities. Each genus comprised one species that has successfully invaded New Zealand grasslands and one species that was introduced but has not been invasive in New Zealand. In an among-species approach, pre-adaptation was tested by comparing germination success of native (European) origins of all four species in relation to their different invasive success in New Zealand. In a within-species comparison, native (European) and exotic (New Zealand) origins of the two invasive species were compared to test for local adaptation to UV-B in the invaded range. In both approaches, UV-B radiation inhibited the germination success of all study species. However, the comparison of invasive and non-invasive species of the two genera showed no UV-B-specific pre-adaptation of invasive species to high

UV-B intensities. Higher germination success of invasive species probably led to an establishment advantage during colonization of the invaded range. Although local adaptation of exotic populations to UV-B could not be demonstrated in the within-species approach, a genetic shift in germination velocity between native and exotic origins was found. These differences may be ascribed to other relevant environmental factors, e.g. overall irradiation and drought, inducing similar plant responses as under UV-B radiation.

Keywords

Congeneric species pairs, *Echium*, invasive vs. non-invasive species, local adaptation, native vs. exotic origins, pre-adaptation, *Verbascum*

Introduction

In the course of globalization, a plethora of species have managed to overcome geographic barriers and to successfully establish wild populations in the new environment. Most notably, this can be observed in previously isolated systems, such as Australia, New Zealand, Madagascar and other islands (Loope and Mueller-Dombois 1989). Following the framework for biological invasions proposed by Blackburn et al. (2011), the environmental conditions in the invaded range represent an important barrier a species has to overcome upon arrival in a new region before it can become invasive. One of the most prominent research goals in invasion ecology is to identify which plant species have the potential to respond successfully to novel environmental conditions in new habitats and which mechanisms are underlying the success or failure of invasions. Two mechanisms that might apply non-exclusively have been repeatedly addressed, i.e. the concept of pre-adaptation including species sorting following environmental filtering (Theoharides and Dukes 2007, Shine et al. 2011) and the role of adaptive evolution following natural selection (Prentis et al. 2008, Buswell et al. 2011). In fact, some species are pre-adapted to become invasive as they have evolved traits in their native regions that confer an advantage in the introduced range (Fridley 2013). In contrast, some species evolve new traits in response to new environments (Erfmeier et al. 2011). Evidence for pre-adaptation can be derived from comparing pairs of invasive and non-invasive species within a genus (Schlaepfer et al. 2010, van Kleunen et al. 2011), whereas more recent local adaptation needs to be tested in within-species comparisons of native and exotic origins (Kawecki and Ebert 2004).

In the last decades, many studies have attempted to identify characteristics that explain species' invasiveness. Frequently evoked characteristics are a large native range and the ability of fast dispersal, as induced by short generation times, high seed production, low seed weight and long seed persistence (Rejmánek and Richardson 1996, Dukes and Mooney 1999). Especially reproduction-related traits seem to be closely connected to the establishment success of invasive species. Species equipped with such a set of characteristics are considered predisposed with respect to successful colonization of new environments. Testing for effects of pre-adaptation in plant invasions relies on comparing congeneric species with different invasive potential. For example, a comparative study including 14 congeneric pairs of species native to Europe and inva-

sive in the US revealed faster germination, higher productivity and a higher proportion of flowering plants of the invasive species than of their native congeners (Schlaepfer et al. 2010). Furthermore, invasive *Impatiens* species displayed a shorter stratification period than their native congeners (Perglová et al. 2009). In particular, higher germination rates of invasive species compared to non-invasive congeners have been identified in numerous studies (Cervera and Parra-Tabla 2009, Perglová et al. 2009).

However, germination patterns have also been shown to differ between exotic and native populations, thereby providing evidence for the alternative explanation of local adaptation taking place in the invaded range. There are some examples of herbaceous and woody plants that indicate lower germination rates of native populations compared to exotic ones (e.g. Kudoh et al. 2007, Beckmann et al. 2011, Hirsch et al. 2012), whereas other studies reported differences in germination velocity (Erfmeier and Bruelheide 2005). Hierro et al. (2009), for example, were able to link different germination patterns of native and exotic populations to regional climatic conditions. Such findings could represent non-random introductions of faster-germinating populations. However, such evidences might also indicate genetic differentiation in germination characteristics between native and exotic populations that resulted from selection processes during introduction (Bossdorf et al. 2005).

Invasive species have to face several biotic and abiotic selection factors in the invaded range, such as competition, herbivory, soil properties and climatic conditions (Erfmeier 2013). Nevertheless, the impact of growth-limiting high UV-B levels on invasion processes has rarely been evaluated to date, although UV-B radiation is distinctively higher in the highly-invaded regions of the southern hemisphere such as in Australia or New Zealand (McKenzie et al. 2007) compared to the origin of many invasive plants.

Early stages of plant development and seedling establishment are particularly sensitive to biologically effective UV-B radiation including metabolic limitations or DNA damage, when appropriate protection measures are not yet fully developed. In particular, reduced seedling biomass, inhibited hypocotyl or root development and growth abnormalities (e.g. shoot curvature) have been observed in response to high UV-B intensities (Krizek 1975, Tevini et al. 1983, Tosserams et al. 1997, Dai and Upadhyaya 2002). In addition, species differ in their responsiveness to UV-B radiation. Musil (1995), for example, described higher UV-B resilience for monocotyledons than for dicotyledons. Evidence of UV-B-induced inhibition of germination has been provided by several studies to date (Tevini et al. 1983, Tosserams et al. 1997, Dai and Upadhyaya 2002). There is also clear evidence for inter- and intraspecific differences in UV-B sensitivity in plants (Hofmann and Campbell 2011). However, UV-B sensitivity of invasive species and the consequences for establishment and spread of populations have been studied only rarely. One of the few examples has revealed differential UV-B effects on later stages of growth in a comparison of populations originating from the native and exotic provenances of *Hieracium pilosella* L. (mouse-ear hawkweed, Beckmann et al. 2012). For example, UV-B radiation caused a decreased belowground biomass and an inhibited vegetative reproduction of *H. pilosella* individuals, as well as significant alterations in leaf morphology.

Hence, we conducted a germination experiment comparing pairs of invasive vs. non-invasive species of the genera *Echium* and *Verbascum*, and native vs. exotic origins of the respective successful invader. We tested for pre-adaptation to high UV-B levels in native populations of invasive species as compared to congeneric non-invasive species, and for effects or more recent evolutionary processes, that may hint at local adaptation of exotic populations from New Zealand in the germination responses. We addressed the hypotheses that a) UV-B radiation inhibits germination success in plants and b) that invasive species and exotic populations show higher germination success in comparison to non-invasive species and native populations of invasive species, respectively. In a first analysis, we compared the germination success in response to high UV-B radiation of species native to Europe that are currently invading New Zealand grasslands with non-invasive congeners that, as yet, have failed to spread to a similar degree in New Zealand. In a second analysis, we tested for within-species differentiation among native and exotic origins. Accordingly, we included seeds of native (European) and exotic (New Zealand) populations of *Echium vulgare* L. (blueweed) and *Verbascum thapsus* L. (common mullein), as well as native (European) populations of their non-invasive congeners *Echium plantagineum* L. (Paterson's curse) and *V. nigrum* L. (dark mullein), respectively. This is the first study addressing the role of UV-B for seedling emergence and establishment for plants invasive in the southern hemisphere.

Methods

Study species

We used seeds of two pairs of congeneric species of the genera *Echium* (Boraginaceae) and *Verbascum* (Scrophulariaceae). All four study species are native to Central Europe or distributed in Eurasia and typical components of dry grasslands and ruderal habitats. They are characterized by high drought tolerance and a strong prevalence in open, unshaded habitats (Gross and Werner 1978, Klemow et al. 2002, Sheppard and Smyth 2012). The species of both genera are monocarpic and generally biennial and develop a rosette in the first growing season, whereas a flowering stem sprouts in the second year. In all species, the upper leaf surface is typically piliferous, i.e. *Verbascum* plants are densely covered with woolly, branched stellate trichomes (Gross and Werner 1978), whereas *Echium* leaves are equipped with unicellular, bristly hairs with silicified or calcified cell walls (Möhl 2011). Within each species pair, one species was classified as 'invasive' in the South Island of New Zealand, whereas the other species was considered 'non-invasive' (see Suppl. material 1). For the among-species approach, all European seeds, i.e. native origins, of the invasive and the non-invasive species were investigated. In addition, for the within-species approach, European native origins were compared with New Zealand seeds of exotic origins for *Echium plantagineum* and *Verbascum thapsus*. In total, this resulted in a set of six origins to be tested. Each origin was represented by four populations (except for European seeds of *E. plantagineum*:

n=3 and New Zealand seeds of *E. vulgare*: n=5), i.e. a total of 24 populations. Seeds of the invasive species – *E. vulgare* and *V. thapsus* – were collected in 2011 in grasslands and ruderal habitats between 65–718 m a.s.l., whereas seeds of the non-invasive species – *E. plantagineum* and *V. nigrum* – were obtained from commercial seed companies (for details see Suppl. material 1).

Experimental design and data collection

The germination experiment was conducted at Lincoln University in Lincoln, Christchurch (New Zealand) in March 2012, in a walk-in growth chamber (Type PGV36, Conviron). The UV-B treatment was induced by twelve UV-B tubes (UVB 313 EL, Q-Lab Corporation), which were installed in addition to a standard set of illuminants. Each UV-B tube was enveloped with UV-B-permeable cellulose acetate filter to exclude undesired wavelengths (i.e. UV-C). During the experiment UV-B intensity was measured continuously in the growth chamber by a UV-B sensor, thus, an electronic feedback system kept the intensity constant (Hofmann and Campbell 2012). The irradiance levels were $38.2 \text{ kJ m}^{-2} \text{ d}^{-1}$ biologically effective UV-B, normalised to 300 nm (Caldwell 1971). Half of the seeds were exposed to UV-B by using one frame with six UV-B tubes, while the other half was placed below the second frame where the UV-B tubes were switched off. We installed an UV-B impermeable Mylar® curtain in the middle of the growth chamber to apply UV-B-radiation exclusively to one section of the chamber. Photosynthetically active radiation and UV-B radiation was applied 16 hours a day. During the whole experiment the temperature within the chamber was set at 23 °C with a relative humidity of 95 %.

The germination test was done in seedling trays (i.e. plots) arranged in a split-plot design. Eight seedling trays, filled with sterilized, finely granulated substrate, were each sub-divided in 24 quadrats (5cm × 5cm) being separated aboveground. For origins of *Echium* and *Verbascum*, 12 and 25 seeds per population, respectively, were sown in each quadrat. Each of the 24 populations was sown once per plot, i.e. each population was replicated eight times in total. Half of the plots were exposed to UV-B exposure, whereas the other four plots served as a control, i.e., no UV-B. The plots were randomly positioned within the two UV-B treatments and randomization was repeated twice during the experiment, including reassignment of UV-B applications to the two sections within the chamber.

The seeds were sown on the wet substrate and slightly pressed in. The trays (i.e. plots) were placed in tubs, filled with water, to keep the substrate moist. In addition, the trays were sprayed with water every second day to prevent them from drying out. From the third day of the experiment onwards, the number of germinated seeds was initially recorded daily and later every other day (ten times in total). The last germination event was monitored on day 17. Newly emerged seedlings and cases of seedling mortality were assessed visually. At the end of the experiment, we counted the final number of seedlings in each quadrat and determined the germination success.

Statistical analysis

For data analysis, linear mixed models were applied in SAS 9.2 (SAS Institute Inc. 2000) using the procedure ‘mixed’ and type 3 sum of squares. Due to a lack of normal distribution, the data for germination success were analyzed following square root transformation (as recommended by Zuur et al. 2009).

Data were analyzed according to an orthogonal design reflecting the hypotheses by first comparing invasive and non-invasive species (among-species approach: $n = 15$ populations) and secondly, comparing native and exotic origins of the two invasive species *E. vulgare* and *V. thapsus* (within-species approach: $n = 17$ populations).

For the among-species approach, germination data of native populations of all four investigated species ($n = 120$ quadrats) were included in the statistical analysis, testing maximum germination success as a response variable. The model included ‘genus’ (*Echium*, *Verbascum*), ‘status’ (invasive, non-invasive) and ‘treatment’ (UV-B, no-UV-B) as fixed factors, whereas ‘treatment \times plot’ and ‘genus \times status \times population’ were considered random factors. In order to test for differences in germination velocity, a repeated measures analysis was done by adding ‘days’ (i.e., the date of seedling census) as a continuous variable and ‘genus \times status \times treatment \times unit (location in a certain quadrat)’ as a random factor to the model.

For the within-species approach, germination data of native and exotic populations of the invasive species *E. vulgare* and *V. thapsus* were tested for potential differences in UV-B tolerance ($n=136$ quadrats), applying the same model. Therefore, maximum germination success was tested in a model containing ‘species’ (*E. vulgare*, *V. thapsus*), ‘origin’ (DE, NZ) and ‘treatment’ (UV-B, no-UV-B) as fixed factors. ‘Species \times origin \times population’ and ‘treatment \times plot’ were included as random factors. Again, a repeated measures analysis was done for all ten censuses during the experiment, using a similar mixed model but also including ‘days’ as a continuous variable and ‘species \times origin \times treatment \times unit’ as a random factor.

All figures were produced with R 3.0.0 (R Core Team 2013).

Results

The among-species approach: invasive vs. non-invasive species

Both genera differed significantly in germination success with a higher maximum germination of the *Verbascum* species ($p < 0.001$, Fig. 1, Table 1). There was a significant effect of status, i.e., in both genera the invasive species – *E. vulgare* and *V. thapsus* – showed a higher final germination than the non-invasive species ($p=0.006$, Fig. 1a). The non-significant interaction effect of ‘genus’ and ‘status’ confirmed the same trend in both genera (Table 1). UV-B radiation caused an overall reduction of germination success in *Echium* and *Verbascum* species ($p=0.022$, Fig 1b). However, a significant

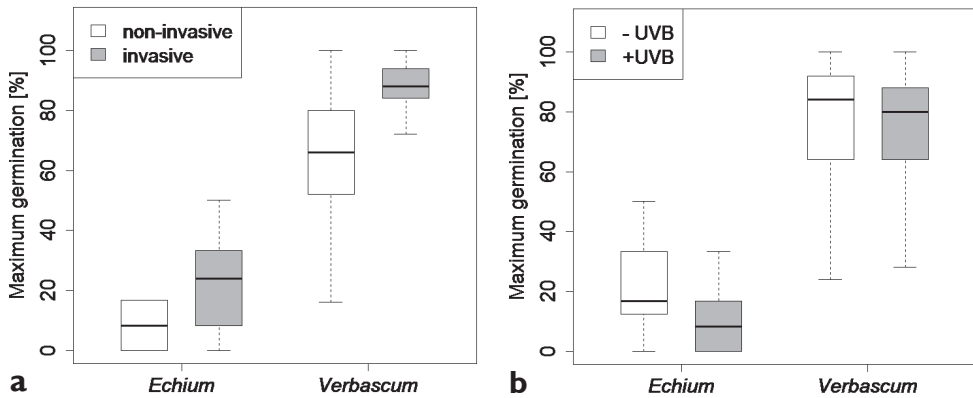


Figure 1. Maximum germination in the among-species approach testing for pre-adaptation of *Echium* and *Verbascum* seeds. **a** of invasive (grey) and non-invasive (white) species and **b** in response to UV-B radiation (grey) and no UV-B radiation (white) treatment.

Table 1. Results of the mixed model analysis of the among-species approach testing for pre-adaptation. Degrees of freedom (df_N = numerator, df_D = denominator), F-statistics (F) and significance values (p) are given. ‘Plot \times treatment’ and ‘genus \times status \times population’ interactions were considered random effects and a Z-value instead of F-value is provided. Bold numbers indicate significant p-values ($p < 0.05$).

Source	df_N	df_D	F/Z	p
treatment	1	6	9.33	0.022
genus	1	11	86.77	<0.001
status	1	11	11.35	0.006
genus \times status	1	11	0.70	0.421
genus \times treatment	1	95	11.41	0.001
status \times treatment	1	95	0.36	0.551
genus \times status \times treatment	1	95	0.44	0.507
treatment \times plot			0.56	0.286
genus \times status \times population			2.03	0.021

‘genus \times treatment’ interaction effect indicated a stronger decrease of germination by UV-B in the genus *Echium* than in the genus *Verbascum* ($p=0.001$, Fig 1b). There was no evidence for a different UV-B tolerance of both statuses, i.e. between invasive and non-invasive species (Table 1).

Repeated measures analysis confirmed these effects over time (Table 2). In addition, significant two- and threefold interactions among ‘days’, ‘genus’ and ‘status’ suggested a different course of germination of the two statuses in both genera over time ($p=0.003$, Fig. 2). Thus, *V. thapsus* and *E. vulgare* did not only attain higher germination rates but also germinated more quickly.

Table 2. Results of the repeated measures analysis of the among-species approach testing for pre-adaptation. Degrees of freedom (df_N = numerator, df_D = denominator), F-statistics (F) and significance values (p) are given. ‘Treatment × plot’, ‘genus × status × population’ and ‘genus × status × treatment × unit’ interactions were considered random effects and a Z-value instead of F-value is provided. Bold numbers indicate significant p-values ($p < 0.05$).

Source	df_N	df_D	F/Z	p
treatment	1	6	0.45	0.529
genus	1	11	39.20	<0.001
status	1	11	4.67	0.054
genus × status	1	11	0.32	0.584
genus × treatment	1	95	0.32	0.575
status × treatment	1	95	0.00	0.986
genus × status × treatment	1	95	0.65	0.421
days	1	1072	675.56	<0.001
days × genus	1	1072	44.62	<0.001
days × status	1	1072	6.42	0.011
days × treatment	1	1072	3.83	0.051
days × genus × status	1	1072	9.11	0.003
days × genus × treatment	1	1072	6.98	0.008
days × status × treatment	1	1072	0.09	0.764
days × genus × status × treatment	1	1072	0.01	0.921
treatment × plot			-	-
genus × status × population			2.18	0.015
genus × status × treatment × unit			4.42	<0.001

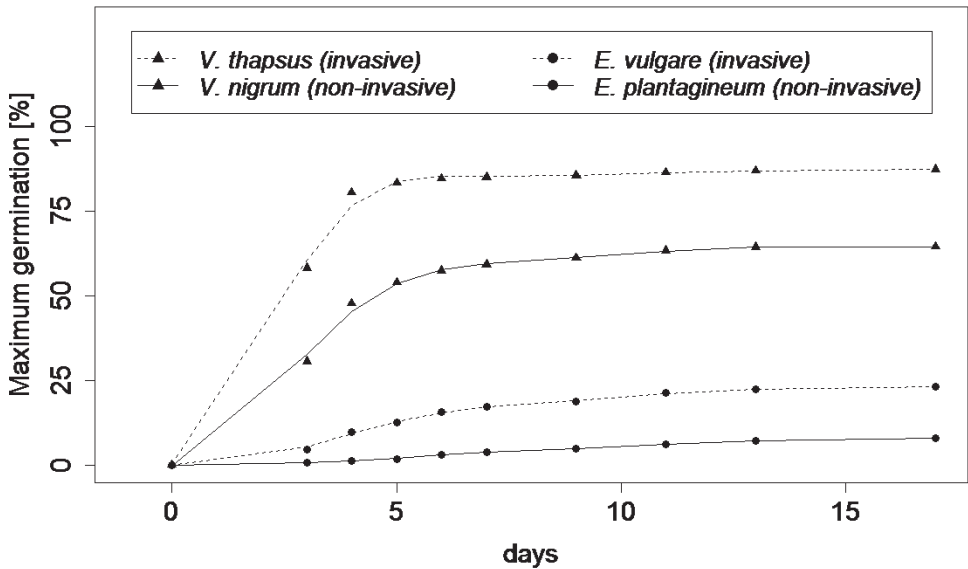


Figure 2. Germination development in the among-species approach testing for pre-adaptation of exotic (dashed line) and native species (solid line) of the genera *Echium* (circle) and *Verbascum* (triangle).

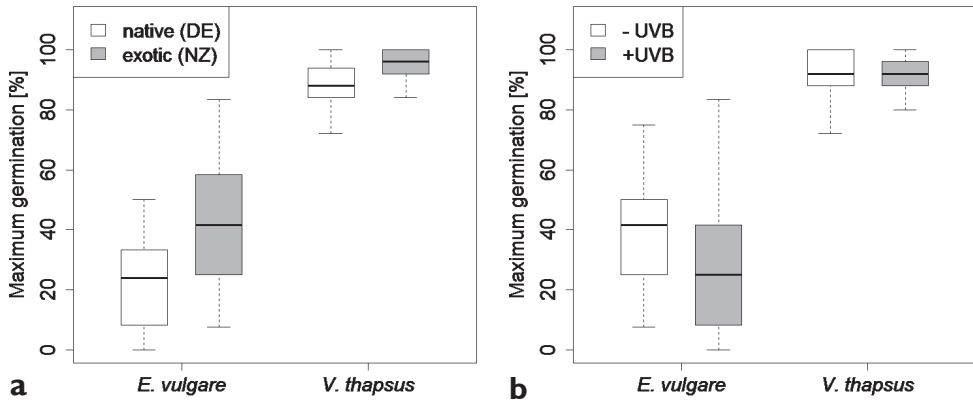


Figure 3. Maximum germination in the within-species approach testing for local adaptation of *Echium vulgare* and *Verbascum thapsus* seeds. **a** of New Zealand (grey) and German (white) populations and **b** in response to UV-B radiation (grey) and no UV-B radiation (white) treatment.

The within-species approach: Native vs. exotic origins

V. thapsus had a higher maximum germination success than *E. vulgare* ($p < 0.001$, Fig. 3). For both species, a significantly higher maximum germination was observed for exotic populations from New Zealand as compared to native German origins ($p = 0.004$, Fig. 3a). The interaction between ‘species’ and ‘origin’ marginally failed to be significant but indicated a larger difference between native and exotic populations for *E. vulgare* than for *V. thapsus* by trend ($p = 0.051$, Fig. 3a). Overall, the UV-B treatment did not lead to a significantly reduced germination success ($p = 0.055$, Fig. 3b). However, a significant ‘species \times treatment’ interaction showed a stronger effect of UV-B radiation on germination success of *E. vulgare* than of *V. thapsus* ($p = 0.003$, Fig. 3b). A difference between native and exotic populations in response to high UV-B levels, as would have been displayed by a significant ‘origin \times treatment’ interaction, was not found (Table 3).

The repeated measures analysis confirmed these results over time and revealed a faster germination of *Verbascum* seeds compared to *Echium* seeds and also a higher germination velocity of exotic origins of both species than native ones (Table 4). A significant threefold interaction effect of ‘days’, ‘species’ and ‘origin’ indicated a larger difference between native and exotic populations for *E. vulgare* than for *V. thapsus* ($p = 0.043$, Table 4).

Discussion

The role of pre-adaptation

The among-species approach showed a higher maximum germination rate of invasive species compared to non-invasive congeners and an overall inhibiting effect of UV-B

Table 3. Results of the mixed model analysis of the within-species approach testing for local adaptation. Degrees of freedom (df_N = numerator, df_D = denominator), F-statistics (F) and significance values (p) are given. ‘Treatment \times plot’ and ‘species \times origin \times population’ interactions were considered random effects and a Z-value instead of F-value is provided. Bold numbers indicate significant p-values ($p < 0.05$).

Source	df_N	df_D	F/Z	p
treatment	1	6	5.64	0.055
species	1	13	135.30	<0.001
origin	1	13	12.25	0.004
species \times origin	1	13	4.63	0.051
species \times treatment	1	109	8.97	0.003
origin \times treatment	1	109	1.21	0.275
species \times origin \times treatment	1	109	2.26	0.136
treatment \times plot			-	-
species \times origin \times population			1.81	0.035

Table 4. Results of the repeated measures analysis of the within-species approach testing for local adaptation. Degrees of freedom (df_N = numerator, df_D = denominator), F-statistics (F) and significance values (p) are given. ‘Treatment \times plot’, ‘species \times origin \times population’ and ‘species \times origin \times treatment \times unit’ interactions were considered random effects and a Z-value instead of F-value is provided. Bold numbers indicate significant p-values ($p < 0.05$).

Source	df_N	df_D	F/Z	p
treatment	1	6	0.23	0.646
species	1	13	85.51	<0.001
origin	1	13	1.22	0.289
species \times origin	1	13	0.18	0.680
species \times treatment	1	109	0.14	0.706
origin \times treatment	1	109	0.02	0.890
species \times origin \times treatment	1	109	0.38	0.542
days	1	1216	899.12	<0.001
days \times species	1	1216	2.14	0.144
days \times origin	1	1216	7.72	0.006
days \times treatment	1	1216	1.00	0.317
days \times species \times origin	1	1216	4.12	0.043
days \times species \times treatment	1	1216	4.84	0.028
days \times origin \times treatment	1	1216	0.33	0.563
days \times species \times origin \times treatment	1	1216	0.17	0.678
treatment \times plot			-	-
species \times origin \times population			2.13	0.017
species \times origin \times treatment \times unit			4.70	<0.001

radiation on germination success independent of the species’ status. This positive association of rapid germination and high germination percentage of seeds of native origins with species’ invasiveness has been also described in preceding studies (Radford and Cousens 2000, Cervera and Parra-Tabla 2009, Schlaepfer et al. 2010). In addi-

tion, a multi-species approach by Kempel et al. (2013), comparing 48 invasive and 45 non-invasive species, revealed the relevance of early life stages characteristics. In their study, in particular, propagule pressure and seed mass appeared to be important for the differentiation among species in order to predict species invasion success. Thus, these reproductive traits are likely to be beneficial characteristics, which directly increase establishment success of invasive species in the invaded range. A faster germination and higher germination rate have been described for invasive species, both in the native and invaded range, compared to the respective non-invasive species (Pyšek and Richardson 2007). Thus, these germination characteristics can be considered a pre-adaptation fostering invasiveness but will also become important traits under selection after the introduction of the species in a new range (Schlaepfer et al. 2010). However, the present study does not give an indication for a pre-adaptation of invasive species to high UV-B intensities, since different germination patterns between the two statuses in response to the UV-B treatment were not detected. This lack of evidence could be a consequence of the low number of congeneric species pairs included in the present study. A multi-species approach can be much more suitable to reveal existing pre-adaptation patterns (van Kleunen et al. 2010). Nevertheless, our findings display an overall negative effect of the abiotic factor UV-B radiation. The inhibiting effect of UV-B radiation on germination observed, both in the among-species and in the within-species approach of this study, has been previously described for several other species. Tevini et al. (1983), for example, confirmed reduced germination under high UV-B levels for three crop species. A similar result was found for *Bromus tectorum* (Dai and Upadhyaya 2002) and *Senecio jacobaea* (Tosserams et al. 1997). Thus, from a physiological perspective, UV-B radiation may indeed act as a selective agent for early life-cycle phases of plants species, even though its differential potential was not able to be shown.

The present study also indicates a stronger inhibition of germination under UV-B for *Echium* species, whereas *Verbascum* seeds, especially of invasive *Verbascum thapsus*, seem to be more UV-B tolerant. The tissues surrounding an embryo might act as UV-B shield, which could be a possible explanation of seed UV-B tolerance (van de Geijn et al. 1993). Various studies characterized the germination of *Verbascum thapsus* as strongly dependent on water, temperature and light (Gross 1980, Baskin and Baskin 1981, Gross and Werner 1982), whereas successful germination of this species is clearly reduced by shading (Semenza et al. 1978, Gross 1980). Possibly, due to the strong light affinity, highly developed photoprotection mechanisms can be assumed for *Verbascum thapsus*.

Evidence of recent adaptation

The within-species approach addressed the role of local adaptation in the invaded range and revealed a higher germination success of exotic origins of the invasive species from New Zealand compared to the native German populations. Again, stronger differences in germination proportions between exotic and native provenances were encountered

for the *Echium* species compared to *Verbascum*, in particular when considering progression with time. Beckmann et al. (2011) confirmed this result for three different exotic grassland species in New Zealand and suggested that local adaptation of germination patterns towards different temperature conditions in the exotic populations might be at play. Our data do not support the idea of a similar adaptation to the environmental factor UV-B radiation, since the maximum germination of both origins was reduced to the same extent by the UV-B treatment. Admittedly, as the seeds used in this study are field-collected or obtained from seed companies, it is hard to disentangle, whether altered germination patterns across ranges are truly adaptive or superimposed by maternal effects (Galloway 2001). Non-genetic effects, i.e. differences in handling or maternal effects have proven to hamper the interpretation of adaptive processes (Kawecki and Ebert 2004). This caveat can be overcome by referring to seed material from F1 offspring of sampled populations in future experiments. Furthermore, the included populations do not represent the broad distribution range of the study species and populations originating from other parts of the native range than sampled may display a different response, thus, making general deductions more difficult. However, differences between invasive and native populations might suggest a genetic shift due to evolutionary processes in the invaded range. Nonetheless, the encountered effects cannot directly be attributed to UV-B radiation, and might also be a consequence of other environmental factors in the habitat, which affect plant individuals in a similar way and cause comparable plant responses: The underlying selection pressure towards higher and faster germination might be caused, e.g., by water supply. In particular drought, which can evoke similar plant responses as UV-B (Tevini et al. 1981, Hofmann et al. 2003), is a factor to be considered, preferably in interaction with UV-B. UV-B effects interact with irradiation intensity and with exposure to drought (Hofmann and Campbell 2011), affecting physiological responses in grassland habitats, and thus the interaction of such factors for germination attributes need to be more critically taken into consideration in future studies.

Conclusions

The present study confirmed a distinctly inhibiting effect of UV-B radiation on germination of the studied species, but did not provide evidence for a UV-B specific pre-adaptation of invasive species or local adaptation of exotic populations to UV-B in the invaded range. The overall reducing effect of UV-B radiation on germination suggests that UV-B represents an important selection factor in the invaded range, and particularly for alien species colonizing new habitats in parts of the southern hemisphere. Possibly, neither pre-adaptation nor local adaptation fully explains the invasive potential of the study species. Instead, the phenotypic plasticity to a broad range of environmental conditions in terms of a ‘general-purpose genotype’, as was already mentioned for *Verbascum thapsus* (Parker et al. 2003), might be of higher relevance (but see: van Kleunen et al. 2011).

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Supplementary material I

Seed origins and population locations

Authors: Maria Hock, Michael Beckmann, Rainer W. Hofmann, Helge Bruelheide, Alexandra Erfmeier

Data type: occurrence data

Explanation note: Overview on seed origins of the study species and locations of the populations used in the experiment. Numbers in brackets indicate the number of populations within the respective group. DE = Germany, NZ = New Zealand, ES = Spain, NL = The Netherlands.

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Research on mutualisms between native and non-native partners can contribute critical ecological insights

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Abstract

Mutualisms are important structuring forces in ecological communities, influencing ecosystem functions, diversity, and evolutionary trajectories. New interactions, particularly between native and non-native species, are globally increasing in biotic communities as species introductions accelerate. Positive interactions such as novel mutualisms can affect the fitness of organisms in invaded communities. Non-natives can augment native mutualism networks, replace extinct native partners, or disrupt native mutualisms. Because they are actively forming or newly formed, novel mutualisms offer a unique opportunity to examine in real time the factors governing early mutualism formation and stability, including frequency-dependent processes and those relying on specific traits or functions. These central ecological questions have been inferred from long-formed mutualisms, but novel mutualisms may allow a glimpse of successes and failures in ecological time with insights into the relative importance of these factors as ecological systems shift. To this end, this commentary addresses how novel mutualisms inform our understanding of mutualism formation, stability, the importance of functional traits, and niche vs. neutral processes, using examples across multiple systems. Novel mutualism research thus far has been largely limited in both questions and ecosystems, but if more broadly applied could benefit both theoretical and applied ecology.

Keywords

Positive interactions, marine, belowground, biological invasions, non-native species, novel mutualisms

Introduction

Mutualisms as important structuring forces

Mutualisms are thought to be ubiquitous across habitat types (Bronstein et al. 2004), driving trait evolution and structuring ecological communities (Bruno et al. 2003; Bronstein et al. 2006). Participating species offer and receive currencies such as protection, nutrients, or transportation. Individual species often interact with multiple partners, creating complex mutualistic networks that can confer resilience to species losses through partner redundancy (Aizen et al. 2012). Mutualisms are now recognized as fundamental to ecological complexity and stability (Montoya et al. 2006).

Ecologically and evolutionarily, mutualisms have shaped many biological invasions and vice versa (recently reviewed by Traveset and Richardson 2014). The importance of mutualisms in facilitating invasion and the potential for mutualism evolution to change as a result of biological invasions are critical areas of study (Kiers et al. 2010; Traveset and Richardson 2014). However, the formation of “novel mutualisms” between native and non-native species also serves as a remarkable opportunity to study the processes governing mutualism formation, failure, and fitness implications. As they become established in ecological communities, non-native species may enter mutualistic networks, potentially altering community dynamics and ecosystem functions (Traveset and Richardson 2014). Fundamental research in ecology has centered on the role of mutualisms in emergent ecosystem properties—that is, how mutualism formation, structure, and stability may have generated the species combinations and interactions observed in ecosystems today. Since this research concentrates on past processes, it is largely inferential, attempting to reconstruct likely pathways and thereby better understand ecosystem function. Two key areas of study include: 1) mutualism formation and stability in the context of partner cheating, opportunism, and partner extinction (Bronstein et al. 2006); and 2) how species’ traits and functions may or may not have predisposed them to participating in mutualisms (e.g., Janzen 1985). This latter line of inquiry examines, in essence, which comes first: participation in mutualisms or the traits that predispose participation, attempting to sort neutral opportunism from niche processes. Novel mutualisms provide an opportunity to examine these critical ecological questions from the opposite end of the process timeline: researchers can examine mutualisms as they form or fail, including the importance of functional traits, niche and neutral processes, and their stability over ecological time.

Because it focuses on early mutualism stages (rather than inferences from later stages), novel mutualism research may improve our understanding of the foundation for mutualisms more generally as well as the implications of potential mutualist introductions. This commentary uses specific examples of novel mutualisms to discuss early mutualism formation and stability, the role of functional traits, and niche vs. neutral processes in community assembly. Novel mutualism research to date has been heavily concentrated in the realms of invasion biology and aboveground, terrestrial ecosystems. We deliberately discuss traits of novel mutualisms across ecological domains to seek broad patterns relevant to these new avenues of research.

A useful definition

Scientific literature uses a variety of definitions for the term “mutualism.” The simplest, conventional definition for mutualism is any interaction between two species that results in fitness benefits for both species (notated as “+/+”) (Bronstein 1994b). This contrasts with antagonistic relationships in which one species benefits and the other is harmed (predation, parasitism, competition: “+/-”) and commensal relationships in which one species benefits while the other is unaffected (“+/0”) (Bronstein 1994b). Examples throughout this commentary reflect this definition of mutualism, although we acknowledge that interactions that encompass facilitation or other indirect benefits may represent nascent points from which more traditional mutualisms may arise.

Box 1. Glossary of terms describing interspecific interactions.

Mutualism: an interaction wherein both sides derive fitness benefits and the interaction occurs at the individual level (“+/+”).

Facilitation: an interaction wherein at least one species derives a fitness benefit and the other side is not detrimentally affected.

Obligate: an interaction necessary for survival of one or both of the species involved. (Antonym: Facultative)

Specific: an interaction in which a species has only a single partner or very small number of partners. (Antonym: General)

Diffuse: an interaction involving many species or a network. (Antonym: Pairwise)

Novel mutualisms as examples of early mutualism formation

Basic evolutionary and ecological research focused on emergent ecosystem properties has explored the factors driving mutualism formation. Benefits of mutualisms can be contextual (Bronstein 2001; Hay et al. 2004), leading to questions about the circumstances and strength of selection required for mutualisms to form (Sachs et al 2004; Foster and Wenseleers 2006) and persist. Species that form novel mutualisms must either be 1) initially capable of surviving and reproducing without mutualism (facultative) or 2) interactors that can form new, necessary mutualisms with little regard for partner identity (generalist) (Brodie et al. 2014). Species requiring mutualism to survive or reproduce may fail to recruit in a new ecosystem early in the invasion process, before novel relationships can develop (Fig. 1; Theoharides and Dukes 2007; Richardson and Pyšek 2012). Consistently, most examples of novel mutualisms are both facultative and generalist (Traveset and Richardson 2014). This parallels our contemporary understanding of most native-native mutualisms (Blüthgen et al. 2007).

Thus, novel mutualisms appear to recapitulate a general mutualism tendency to be facultative, particularly early in the mutualism formation process. Known facultative novel mutualisms include most novel seed dispersal relationships (e.g., Lenda et al. 2012), ant-plant and ant-insect mutualisms (Ness and Bronstein 2004), and a single example of a novel cleaning relationship: ectoparasite removal from non-native cows, *Bos javanicus*, by native birds, *Corvus orru* (Bradshaw and White 2006). Many invasive

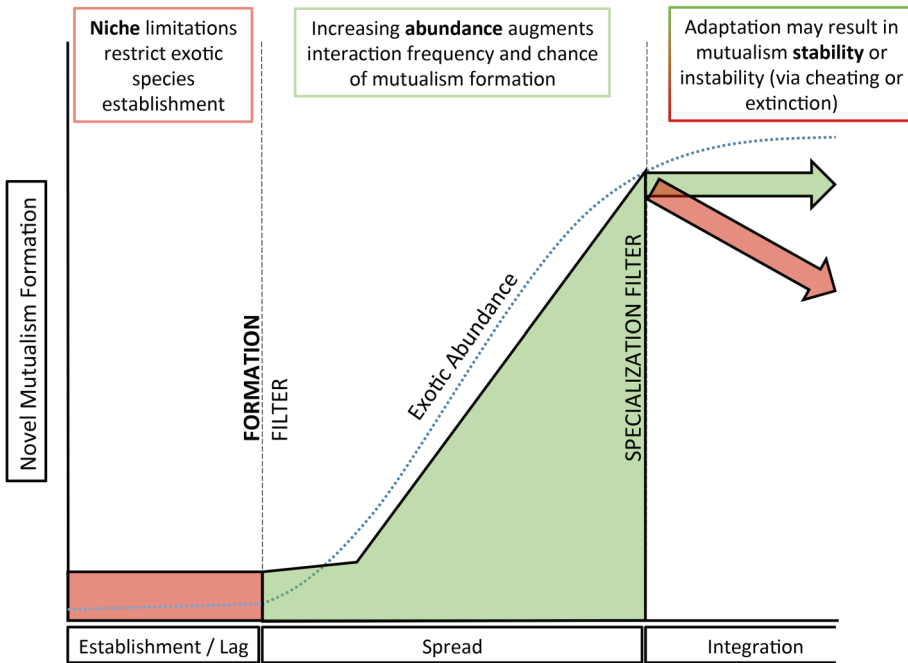


Figure 1. Conceptual figure of the formation and stability of novel mutualisms by niche and neutral processes at different stages of invasion. The x-axis represents three general stages of a biological invasion as necessary for mechanistic purposes, despite substantial debate in the literature about how invasion stages should be delineated. The y-axis represents the formation of novel mutualisms and the dotted line represents the shifting relative abundance of the non-native species. Red areas represent limitation of novel mutualism formation by the process described; green areas represent enhancement or reinforcement by the process described. Hashed vertical lines represent filters: the mutualism formation filter may be imposed by functional traits in the non-native organism or by niche traits preventing mutualism formation. Once the mutualism has formed and the species has spread, mutualism specialization filters may be imposed by strong selection for certain mutualistic traits; increased specialization may impact mutualism stability.

plants appear to be self-compatible or able to reproduce vegetatively, making animal-mediated pollination facultative in these cases (Burns et al. 2011). There are, however, exceptions: several aggressive invasive plants, such as purple loosestrife (*Lythrum salicaria*), Amur honeysuckle (*Lonicera maackii*), and multiflora rose (*Rosa multiflora*), differ from this pattern and demonstrate a stronger dependence on pollinators, partnering with generalist species during establishment (e.g., Jesse et al. 2006). Belowground, formation of novel associations between plants and soil microbes still occur readily (Traveset and Richardson 2014), despite the reduction of benefits in some cases. For example, non-native and native legumes in a Mediterranean shrubland formed partnerships with non-native rhizobia, despite the fact that benefits for native plants from the association were far below those typical of native rhizobial associations (Rodríguez-Echeverría et al. 2012). Similarly, the evolution of reduced mycorrhizal dependency of non-native plants

is evident from field data drawn from different populations of the same plant (Seifert et al. 2009). A notable exception is ectomycorrhizal (EcM) plants invading ecosystems in the Southern hemisphere that rely on mycorrhizas to establish and spread (Richardson et al. 2000). The complementary dependence of non-native soil biota on native host mutualisms is poorly studied. Most soil mutualists (e.g. mycorrhizal fungi, rhizobia, etc.) must form associations to complete their life cycle, although benefits from novel associations for the few EcM species studied may be reduced (Vellinga et al. 2009).

To our knowledge, there are only two documented examples of novel mutualisms in marine systems. One such novel marine mutualism is also facultative. The native sediment dwelling polychaete *Diopatra cuprea* in the mid-Atlantic U.S. incorporates the invasive red alga *Gracilaria vermiculophylla* into its decorated tube caps (Thomsen and McGlathery 2005). The non-native alga is also found unattached, but the stable attachment point of *Diopatra* tube caps increases its longevity and growth rate (Thomsen et al. 2009) and biomass (Byers et al. 2012). Although the nature of the reciprocal benefits of *G. vermiculophylla* to *D. cuprea* have not been investigated, *D. cuprea* generally receives shelter and food from the algae it hosts and favors attaching *G. vermiculophylla* over other native algal species (Thomsen and McGlathery 2005). The other, newly-discovered marine novel mutualism involves a non-native zooxanthella that partners with corals in the Caribbean, with mixed fitness implications: the novel partnership results in increased heat tolerance but reduced calcification for the native partners (Pettay et al. 2015).

Does the lack of other marine examples of novel mutualisms arise from a dearth of research or from a true lack of novel mutualism formation? The few other relevant research attempts in marine systems have failed to detect novel mutualisms. For example, an unusual zooxanthella found in a native Hawaiian coral, once considered a novel mutualism, is instead of cryptogenic origin (LaJeunesse et al. 2008). A search for novel coral-endosymbiont mutualisms in Jamaica was fruitless, despite a high probability of detection due to both the long-term establishment of an Indo-Pacific non-native coral and its common acquisition of endosymbionts by horizontal transfer in its native range (LaJeunesse et al. 2005). Observations of the invasive lionfish, *Pterois volitans*, loitering at reef cleaning stations throughout its invaded range in the Caribbean appear to be the behavior of a sit-and-wait predator (Côté and Maljkovic 2010) rather than the formation of a novel mutualism. In spite of these notable absences, however, a considerable increase in marine novel mutualism research is needed before it will be possible to conclude that the formation of novel marine mutualisms is rare and to consider the implications of such rarity for marine mutualism formation. Observation of this research gap may in itself increase awareness and detection.

Informing our understanding of mutualism stability using novel mutualisms

Research on novel mutualisms may also help us understand why some early mutualisms fail while others result in long-term associations. A focus of mutualism stability research is partner fidelity in the presence of “cheaters” (Ferriere et al. 2002). When cheating

predominates, mutualisms can become parasitic, with only one partner benefiting (Bronstein 1994a). Mutualism networks containing “cheating” partners, however, may occur only when beneficial partners are also present (Weyl et al 2010). Species that are generalist, partnering with many mutualistic partners, are likely to obtain mutualistic currencies from some partners, and thus to continue to participate in mutualisms even in the presence of cheaters. Mutualism stability is also a consideration when mutualisms become more specialized or obligate over time and partners are at risk of extinction (Thébault and Fontaine 2010). Once again, mutualism stability may be enhanced by generalist partnerships, wherein each species has multiple partners so loss of a specific partner carries reduced impact. Mutualism specialization can evolve when fitness enhancements offset the enhanced probability of mutualism disruption (Schemske and Horvitz 1984). As early stage mutualisms, novel mutualists may derive lower levels of benefit on a per-mutualistic-interaction basis, but could be more stable than native mutualisms that have formed over a longer timeframe and more commonly exhibit specialization and cheating.

Since generality logically confers increased stability and reduces dependence on cheating or at-risk partners, it is unsurprising that mutualism network literature has demonstrated that generality is extremely common (e.g., Bascompte and Jordano 2014). Certain non-native mutualists appear to take this generality to an extreme, acting as “super-generalists” in novel mutualism networks (Olesen et al. 2002). Studies focused on individual species and their interactions have similarly detected high generality in novel mutualisms. For example, non-native ants that have been reported to partner with native plant species are generalists interacting with a wide variety of species across a global range (Ness and Bronstein 2004). Tending mutualisms between introduced ant species and native aphids are often similarly diffuse, with each ant species interacting with a number of aphid species (Ness and Bronstein 2004). Non-native species acting opportunistically can be generalist mutualists under some contexts and cheaters under others; the non-native Japanese white-eye (*Zosterops japonicus*), for example, nectar-robs some endemic Hawaiian plant flowers but pollinates others (Aslan et al. 2014). Recent evidence that Douglas fir (*Pseudotsuga menziesii*) invading New Zealand can readily form mutualisms with a diversity of native ectomycorrhizal fungi (Moeller et al. 2015) runs counter to other ectomycorrhizal invasive plants in the Southern hemisphere whose associations appear restricted to cosmopolitan or co-invading fungi (Nuñez et al. 2009; Dickie et al. 2010; Bogar et al. 2015). While no research has explored the network impact or stability of these interactions, we can logically predict that diverse associations may enhance stability.

Using novel mutualisms to improve our understanding of the importance of functional traits in community composition

Classic research on mutualism formation and stability also quantifies traits of mutualistic partners as potential limiting reagents that have led to the mutualisms present today. Functional traits form the foundation of many mutualistic networks. For exam-

ple, pollination syndromes and functional groups of effective partners can generally be defined by flower morphology, color, and reward type of a plant species (Fenster et al. 2004). Additionally, mutualisms appear to underlie the evolution of remarkable traits (Bronstein et al. 2006) such as unique morphologies and behaviors, effectively expanding the trait-space of a community. New understanding of major ecological processes, such as community assembly and environmental gradient effects, has come from taking a trait-based approach (McGill et al. 2006; Kraft and Ackerly 2014). Examining novel mutualisms through this lens has the potential to be especially revealing about the role of positive biotic interactions in community assembly (Sargent and Ackerly 2008), but this type of approach has thus far primarily investigated the prevalence of negative biotic interactions like interspecific competition and natural enemies.

How then, do newly arriving non-native species fit into an existing landscape of functional traits and functional group partnerships within a community? Trait-based methods can distinguish environmental filtering and community-level niche partitioning (Kraft et al. 2008). Under environmental filtering, trait-space should be constrained within a locale relative to that of the regional species pool, whereas community level niche partitioning occurs when traits are more evenly spaced than would be expected by chance due to interspecific competition (Kraft et al. 2006). If assembly rules are general, non-native species should, in the aggregate, pack accordingly into an environmentally constrained trait-space and/or exhibit divergent traits that may permit the filling of empty niches. Studies provide support for these processes (Ordonez et al. 2010; Tecco et al. 2010). In agreement with niche partitioning, species may be more likely to be invasive if they are functionally distinct from the native community and thus able to occupy vacant niches or possess novel weapons (Callaway et al. 2004). On the other hand, sheer propagule pressure (the abundance of the non-native species) has also been shown to underlie invasiveness (Lockwood et al. 2005).

In contrast to interspecific competition, a strong effect of mutualism on community assembly would select for non-native species with traits that link easily into existing mutualism networks, effectively favoring species that have traits more similar to native mutualists (Fig. 1). Such a signature of positive interactions has been detected in native communities of animal-pollinated plants, in which plants in the family Asteraceae benefit from the pollinator attraction of nearby individuals of closely related species (Sargent et al. 2011). Successfully established non-native species often demonstrate traits that facilitate their entry into existing interaction webs. Among non-native plants, animal-dispersed species include many with fleshy fruits such as berries and drupes that are widely appealing to a diversity of native disperser species (Aslan and Rejmánek 2010). Plant families known for high invasive tendencies tend to have open, accessible, and radially symmetrical flowers (e.g., Asteraceae), able to participate in generalist mutualisms and to partner readily with local pollinators in any given region (Traveset and Richardson 2006). Among non-native animals, highly successful mutualists include seed dispersers, which tend to be generalist feeders able to take ready advantage of available fruit resources (e.g., Foster and Robinson 2007). In most cases, such species join existing suites of seed dispersers to enter diffuse networks of seed dispersal (Aslan

and Rejmánek 2010). Most invasive plants also readily form belowground symbioses, despite the fact that not providing resources to soil biota should give invaders a distinct advantage (e.g. *Brassica* spp., Traveset and Richardson 2014). Interestingly, reduced mycorrhizal dependency of *Hypericum perforatum* in its invaded range is strongly correlated to specific changes in root architecture (Seifert et al. 2009). However, it is unclear whether other functional traits generally predispose non-native species to novel belowground mutualisms. Novel mutualisms offer the opportunity to describe the nature of the community filter at the front end of the mutualism formation process (Fig. 1).

The role of neutral and niche processes in novel mutualisms

As incipient partnerships, novel mutualisms also offer a unique opportunity to assess the relative importance of interaction frequency as driven by abundance, a neutral process, with that of adaptive traits that provide specific mutualistic benefits defined by the niches of their potential partners (Kaiser-Bunbury et al. 2014). This comparison is particularly valuable given the rapid increase in abundance of non-native species that become invasive, and the relative importance of these two mechanisms may be dictated by the particular stage of spread of a non-native species (Fig. 1).

The importance of niche and neutral processes in novel mutualisms could change during the invasion process as a non-native species initially establishes at low density, spreads and often increases in abundance, and ultimately is integrated into the community. As outlined above, non-native species requiring specific mutualisms are unlikely to establish because they may not find suitable partners. This limitation is largely niche-based. After establishment, non-native species that become problematically invasive may be present at much higher abundance than native partners. While an abundant non-native can reduce the stability of native mutualisms (Aizen et al 2008; Kaiser-Bunbury et al. 2011), high abundance also increases the frequency with which native mutualists encounter non-native species rather than native species, increasing the number of chances for a novel mutualism to form. For example, severe invasions by knapweed (*Centaurea stoebe*) and leafy spurge (*Euphorbia esula*) provide abundant hosts for native AM fungi, ultimately increasing mycorrhizal abundance and diversity (Lekberg et al 2013). Likewise, heavy infestations of yellow starthistle (*Centaurea solstitialis*) represent a large resource visited by a wide diversity of native pollinators (McIver et al. 2009). If the relative abundance of these non-natives remains high, native species that are able to gain benefit from novel partnerships are likely to have greater success, thereby promoting these novel mutualisms. In this stage, the abundance of the non-native drives mutualism formation and stability, in a neutral process. By contrast, in systems where non-natives established long ago and become integrated into the community, traits that enable their partners to maximize benefit from these relationships should be favored by natural selection and lead to niche-based adaptation. It is also possible that adaptation of native partners to a non-native species may result in cheating or a rise in extinction probability if associating with the non-native species results

in dependence on an unreliable partner, a process typical of an evolutionary dead-end (Schlaepfer et al. 2002).

The relative importance of neutral vs. niche processes may also be explored through mutualistic network models. For example, asymmetric specialization or nestedness is a pervasive feature of known mutualistic networks (Vázquez and Aizen 2004; Bascompte and Jordano 2014; but see Blüthgen 2010) in which the abundant, generalist species in one trophic group (e.g., plants) preferentially interact with many rare, specialist species in the other trophic group (e.g., pollinators) and vice versa. “Forbidden links” in mutualistic networks result from niche differences, when species do not overlap in space or time (e.g., a plant flowers and senesces before an insect emerges, *sensu* Bascompte and Jordano 2014). Non-native species, particularly after establishment, may be present across more seasons and in more locations than natives, reducing the occurrence of “forbidden links” in the community. For example, *Calotropis procera* flowers and fruits continuously in invaded Brazilian shrublands compared with a 2-6 month window in its native range, potentially bridging the phenological gap between native species that often flower in either the dry or wet season (Sousa Sobrinho et al. 2013). Similarly, the non-native tree *Ligustrum lucidum* presents food at a different season than its co-occurring native fleshy-fruited trees in Argentinian dry forests, providing frugivores with a fruit resource in an otherwise resource-poor season (Ferreras et al. 2008). Thus, non-native species that become abundant may serve as constant and abundant partners for native mutualists, bridging modules in mutualistic networks that are separated by space or time (Olesen et al. 2007). The inclusion of abundant non-natives into native mutualism networks does appear to shift the behavior or traits of native mutualists (Olesen et al. 2002; Aizen et al 2008; Kaiser-Bunbury et al 2011). Clearly, the relative importance of niche and neutral processes in mutualisms is a topic that requires greater investigation, and novel mutualisms present such an opportunity.

Tackling new questions and new systems with novel mutualism research

As our discussion illustrates, novel mutualisms may serve as prototypes for the study of mutualism formation and thresholds in mutualism strength and exploitation. Using novel mutualisms as model systems to better understand how species enter and establish in communities may help advance basic research and fill current understanding gaps. Because they are widespread and dynamic, novel mutualisms are promising focal points for a diversity of outstanding basic questions (Table 1), and such work may build on the strong foundation in novel mutualism research established by scientists working to understand the importance of positive interactions in a context of biological invasions (e.g., Richardson et al. 2000; Klironomos 2003; Ness 2003; Traveset and Richardson 2006; Aizen et al. 2008; Kaiser-Bunbury et al. 2011; Rodríguez-Echeverría et. al. 2012). Our hope with this Commentary is to stimulate interest in novel mutualisms by researchers working across systems and subdisciplines. A deeper understanding of the role of novel mutualisms in marine systems is a particular need. The history of ecology as a discipline

Table 1. Examples of questions in ecology and evolutionary biology that could be addressed with novel mutualisms as focal systems.

Research questions	
<i>Basic ecology</i>	
•	Do novel mutualisms promote stability, resilience, and diversity in ecological communities, as native-native mutualisms appear to do?
•	Do mutualistic networks containing non-native species demonstrate the same architectural traits or level of stability as native networks?
•	Do novel mutualisms result from availability of open ecological niches?
•	Might specialization, at the level of the non-native or native interactors, dictate whether novel mutualisms can form?
•	When non-natives establish in interaction networks, to what degree and in what ways are they redundant with native participants?
•	Are some types of interaction networks more easily invaded?
•	Are invaded networks more or less resilient to future extinctions than non-invaded networks?
<i>Evolutionary biology</i>	
•	What can the formation of novel mutualisms—where coevolutionary history is lacking—tell us about mutualism evolution?
•	Do non-native mutualists impose or respond to the same selective pressures as native mutualists, and what are the evolutionary implications of novel mutualisms for native species?

has included examples of generalizable concepts developed in marine systems (e.g., keystone species, Paine 1969; trophic cascade research, Strong 1992). If a true dearth of novel ecosystems in marine systems exists, the development of theory surrounding their absence may help us to better understand fundamental differences among systems.

A diversity of modern research challenges may benefit from a better understanding of mutualism formation and stability and of the roles of functional traits and niche and neutral processes in emergent ecosystem properties. As one example, we can focus on microbial mutualisms, increasingly being recognized as critical players in a wide diversity of previously-unknown contexts (Redford et al. 2012; Sikes et al. 2012). Ecological research on gut and cutaneous microbial mutualisms in animals is still in its infancy, particularly compared to that of endophytic plant mutualists (McFall-Ngai 2008). Mutualisms with microorganisms include diffuse (e.g., lichens; O'Brien et al. 2005), generalist (e.g., some gut bacteria; Montalto et al. 2009), and specialized (e.g., endophytes; Arnold and Lutzoni 2007) partnerships. Most animal-microbial symbioses are thought to be obligate mutualisms (e.g., some *Wolbachia*-host relationships; Kozek and Rao 2007), but microbial invasions are likely rampant and may follow similar rules as invasions by macrobiota (Litchman 2010). Understanding these invasions is more than academic; novel microbial mutualisms may play an increasingly important role in conservation. For example, the emerging fungal pathogen *Batrachochytrium dendrobatidis* is causing global declines in amphibian populations, stimulating conservation research into the potential for inoculation of healthy amphibians with protective skin microbiota in order to reduce susceptibility to the pathogen

(Daskin and Alford 2012). Given the enormous diversity of microorganisms, research in this field holds much promise (McFall-Ngai 2008) and provides a potential proving ground for many of the predictions of this Commentary.

Conclusion

Biological invasions will continue to foment novel mutualisms in every system, with unforeseen consequences for native communities. These organisms provide unprecedented opportunity to observe the early stages of interaction development, and to directly observe processes that can be addressed only inferentially using traditional study systems and species. We suggest that novel mutualisms represent an excellent test arena for current understanding of mutualism formation, mutualism stability, functional traits, and niche vs. neutral processes. Novel mutualisms lend themselves to an expanded set of questions and research systems. Expansion and deepening of novel mutualism research can create synergy between invasion biology, basic ecology, and evolutionary biology, to the *mutual* benefit of all three.

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First record of *Aequorea macrodactyla* (Cnidaria, Hydrozoa) from the Israeli coast of the eastern Mediterranean Sea, an alien species indicating invasive pathways

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Abstract

The species of *Aequorea* attract much scientific interest as they contain the unique Green Fluorescent Protein (GFP). In this work we describe for the first time the discovery of a hydrozoan jellyfish belonging to the genus *Aequorea* from the Israeli eastern Mediterranean that contains and exhibits fluorescent protein. Finding *Aequorea macrodactyla* (Brandt, 1835) in the eastern Mediterranean indicates that changes are occurring in the gelatinous fauna of this area. This hydromedusa is known in the seas adjoining the Mediterranean though most of its records are more than four decades old. We examined and identified the newly discovered Israeli *Aequorea* species by combining two phylogenetic systems, traditional morphological phylogeny and molecular phylogenetics. The molecular identification determined that the species is *A. macrodactyla* but with minor genetic differences in the mtDNA 16S gene marker. A 1% difference between the Israeli and the Japanese *A. macrodactyla* was demonstrated, which suggests that the genetic difference between the Israeli and the Japanese population is small but existent. Invasive pathways for this jellyfish were examined by phylogenetic and taxonomic relationships with similar Cnidaria. The results indicate introduction from the Indo-Pacific as invasive pathway, probably by human transportation, and the discovery of *A. macrodactyla* in the eastern Mediterranean Sea could be interpreted as part of the changes in marine biota as a result of cumulative effects of anthropogenic and global changes that affect the eastern Mediterranean basin.

Keywords

Aequorea macrodactyla, hydromedusa, gelatinous fauna, phylogenetic markers, migration of jellyfish, Lessepsian migration, anthropogenic changes

Introduction

The class Hydrozoa, or the superclass Hydrozoa as suggested by Bouillon and Boero (2000), consists of typical carnivores which are considered to be among the most important planktonic and benthic predators (Bouillon et al. 2006). They prey on fish eggs, fish larvae, some marine invertebrates, and on different sizes of planktonic animals (Frost et al. 2012; Laakmann and Holst 2014). The class contains approximately 3800 species worldwide (Schuchert 2014). *Aequorea macrodactyla* (Brandt, 1835) (Fig. 1) belongs to the family Aequoreidae Eschscholtz, 1829 and is one of the 27 accepted species of the genus *Aequorea* Péron & Lesueur, 1810 (Schuchert 2014). *Aequorea victoria* (Murbach & Shearer, 1902) is the most studied species from this genus. For the first time bioluminescence and biofluorescence were described in a jellyfish and green fluorescent protein (GFP) was discovered (Shimomura et al. 1962) in this species. This discovery enabled the utilization of fluorescent protein (FP) in science and medicine and attracted scientific attention to the unique ability of this genus. *Aequorea macrodactyla* is the second species of jellyfish that has been studied for its bioluminescence and GFP, and is used for commercial products (Xia et al. 2002). In this work we present for the first time a hydrozoan jellyfish belonging to the genus *Aequorea* (Fig. 1A) that contains and exhibits fluorescent protein (Figs 1B, 2) in the Israeli eastern Mediterranean basin. Since the discovery of GFP more than fifty years ago (Shimomura et al. 1962) nearly two hundred natural GFP-like fluorescent proteins (FPs) have been described and the FP became an important tool in biological research (Chudakov et al. 2010; Hunt et al. 2012). By using the term “jellyfish” we include the medusa stage of the scyphomedusae from the class Scyphozoa and the hydromedusae from the class Hydrozoa.

Aequorea macrodactyla is a common hydroidomedusa found in the warmer waters of the coastal region of the East China Sea (Xia et al. 2002). Kramp (1956) claimed that *A. macrodactyla* is widely distributed in the Indian and Pacific Ocean from Africa to America and was recorded also at the southern part of the west coast of Africa. Reports of *A. macrodactyla* from the seas adjoining the Mediterranean began with fishery investigations in the years 1937 and 1938, conducted by Dr. H. Blegvad in the Persian Gulf. He collected one specimen of *A. macrodactyla* in this survey. “No previous records of medusa exist from the Iranian Gulf” (Kramp 1956). From the Arabian Sea, *Aequorea macrodactyla* was collected from great depths and also near the shore (Kramp 1965). Schmidt (1973) refers to *A. macrodactyla* existing in the Red Sea, the Gulf of Eilat and the Gulf of Aqaba. Russell reports in 1939 and 1953 finding *A. macrodactyla* in the English Channel (Kramp 1956). Goy et al. (1991) reviewed jellyfish proliferation based on a survey (1969–1989) from the neritic Lebanese waters and suggested that hydro-

graphic conditions and climate are the most determining factors in temporal distribution of jellyfish. They found some *Aequorea* spp. but not *Aequorea macrodactyla*. Turan et al. (2011) report the presence of *Aequorea globosa* Eschscholtz, 1829 for the first time near the coast of Iskenderun Bay in the northeastern Mediterranean and they suggest ship-mediated transport as probable vector. A year later in 2012, Mamish et al. (2012) observed *Aequorea globosa* near the coast of Syria and considered its appearance an indicator of climate change in the eastern Mediterranean marine environment. Gravili et al. (2013) published a comprehensive review to update the diversity poll of species of what is known as a non-Siphonophora Hydrozoa (NSH) in the Mediterranean, which are traditionally studied as a separate group as their polyp and/or medusa stage is distinctive in global distributions (Kramp 1961; Kramp 1965). In this recent work *A. macrodactyla* was absent from the lists of Mediterranean species but mentioned as being present in neighboring areas: the Gulf of Eilat, the Gulf of Aden and the Red Sea (Gravili et al. 2013). The online Encyclopedia of Life shows more up to date information for the locations of *A. macrodactyla* but the specimen closest to the Mediterranean comes from the Indian Ocean dated 23.7.2007 (Encyclopedia of Life 2014).

Boero and Bouillon (1993) state in their study that the fauna of Mediterranean hydromedusae contains 346 species and consists of 19.5% endemic species. The majority, as much as 72.5%, of the Mediterranean species is present in the Atlantic, as well as distributed worldwide, and it can be considered an Atlantic species that could have entered the Mediterranean Sea through the Strait of Gibraltar after the Messinian Salinity Crisis (MSC), also referred to as the Messinian Event. Only 8% are considered Indo-Pacific and these are mainly restricted to the eastern Mediterranean basin. Twenty years later Gravili et al. (2013) presented more or less the same picture for non-siphonophoran hydrozoan fauna distribution in the Mediterranean Sea. From the 400 species they recorded, as much as 68% originate from the Atlantic Ocean, 20% are endemic to the Mediterranean, 8% are Indo-Pacific and the other 4% are defined unclassifiable.

Morphological species identification in hydrozoan taxonomy is difficult for many species and can be misleading as a result of phenotypic variability (Erpenbeck et al. 2005; Laakmann and Holst 2014). Because of this we combined molecular phylogenetics with morphological species identification to achieve the most accurate results. Since molecular phylogenetics by DNA sequences became a main source of information for the clarification of evolutionary relationships (Wörheide and Erpenbeck 2007), we used this tool with three DNA markers. Defining a single general marker for the taxonomic classification of an organism on all taxonomic levels can also be limited and inaccurate (Lunt et al. 1996; Schultz et al. 2005). Thus, in order to achieve better accuracy, multiple genetic markers should be used for research instead of one single genetic marker (Gruenthal et al. 2007). In this study we used genetic methods that are used on the Hydrozoa by comparing DNA sequencing sites from the nuclear large 28S subunit ribosomal DNA (rDNA) genes (Cartwright et al. 2008; Collins et al. 2008; Cantero et al. 2010). We employed two mitochondrial DNA (mtDNA) markers, the cytochrome c oxidase subunit 1 (COI) gene and the large subunit ribosomal RNA (rRNA) gene 16S. The slow evolutionary rates of COI and 16S rDNA in some hydro-

zoans allow them to be useful markers (McFadden et al. 2011; Zheng et al. 2014). The COI is known as the main metazoan barcoding marker, although in hydrozoans the COI gen has scarcely been employed because of the difficulties involved in amplifying DNA fragments using standard primers (Cantero et al. 2010; Schuchert 2010).

The 16S rDNA has been widely accepted for hydrozoan barcoding purposes and is widely used to resolve phylogenetic questions within the Hydrozoa and has been shown as a better marker for barcoding. It is considered to be a gene that is much easier to amplify and is more available in the GenBank database (Cantero et al. 2010; Moura et al. 2011; Laakmann and Holst 2014; Zheng et al. 2014).

By using both methods in this work, molecular phylogenetics sequences as distance-based methods for the evaluation of genetic distances within and between species and morphological species identification, we anticipated improved accuracy in the taxonomic classification for this new discovery of the Israeli eastern Mediterranean *Aequorea* species.

Materials and methods

Aequorea macrodactyla (Brandt, 1835), known also as *Mesonema macrodactyla* Brandt, 1835 was collected near the Israeli shore line at two different locations during the summer of 2013. The first group of specimens was collected in Sdot Yam, GPS position: 32°29'35"N, 34°51'48"E, on 16.6.2013, with an umbrella diameter of 65 mm to 75 mm. Three other groups of *A. macrodactyla* specimens were found and collected at different sites in the Bay of Haifa, GPS general position: 32°52'N, 35°01'E, on 19.12.2013, with an umbrella diameter ranging between 75 mm to 80 mm. A total of 23 specimens were taken to the laboratory for further identification. Finding *A. macrodactyla* concentrations in two different places at a distance of approximately 55 km from each other and half a year apart indicates that this hydromedusa is established in the region. For the species identification of the Israeli *Aequorea*, two phylogenetic systems were combined: traditional morphological phylogenetics and molecular phylogenetics. Morphological identification was executed according to Mayer (1910), Kramp (1956, 1961, 1965), Russell (1970), Bouillon et al. (2004) and Bouillon et al. (2006).

Samples from the collected jellyfish were processed immediately *in situ*, excising sample cuts from the animals and starting with DNA extraction. Total genomic DNA was extracted using the Wizard® SV Genomic DNA Purification System kit (Promega). Other samples were preserved *in situ* in 95% ethanol for further processing in the laboratory. Throughout the work, care was taken to use sterilized tools and containers, and gloves were worn. The genetic classification was done by matching DNA sequences to global and local data to achieve the most accurate species identification for the sampled specimens (Bayha et al. 2010; Pett et al. 2011).

We designed DNA primers (Table 1) for the locally found jellyfish. After some experimenting a suitable marker was determined for the specimens. The DNA concentration was measured with the NanoDrop, using absorption at 260 nm, and the DNA

itself was prepared using the Promega kit for Polymerase chain reaction and sequencing (PCR) amplification and diluted to a final concentration of 2 ng/ μ L. DNA quality was assessed by running samples on 1% agarose gels. All amplifications were carried out in a T100™ Thermal Cycler (BIO-RAD) using GoTaq® Green Master Mix (Promega). PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The sequencing of the specimens was performed by HyLabs sequencing service (Israel).

For the analyses, the sequences were processed and aligned with BioEdit version 7.2.5 (Hall 1999), ClustalX (Larkin et al. 2007), RAxML (Silvestro and Michalak 2012), Nucleotide composition and analyzed with MEGA6. Each phylogenetic tree was processed with MEGA 5.2 and MEGA 6 tools in order to select the best and most appropriate evolutionary phylogenetic model (Tamura et al. 2013).

Results

Systematics

Class HYDROZOA Owen, 1843

Order LEPTOTHECATA Cornelius, 1992

Family AEQUOREIDAE Eschscholtz, 1829

Genus *Aequorea* Péron & Lesueur, 1810

Fig. 1A

Aequorea macrodactyla (Brandt, 1835)

Morphological description. The freshly collected jellyfish were observed carefully in the lab by using: Nikon SMZ100 Binocular, Nikon AZ100 Binocular with DS-Ri1 camera, and Zeiss Imager microscope M2. All specimens have a biconvex lens umbrella (central disc is lens-shaped) of around 20 mm thick and 65 mm to 80 mm in diameter. The stomach is shallow with a diameter of a little less than half of the umbrella, around 30 mm to 38 mm (Fig. 1A). There is an average of 32 straight radial canals (Fig. 1B) and the gonads are linear on both sides of each radial canal (Fig. 1C). The jellyfish have between 10–20 broad marginal tentacle bulbs (Fig. 1A) each with abaxial keel (Fig. 1D). The identification of *Aequorea* spp. can be confusing mainly due to their considerable variability, and misidentification has occurred in the past (Kramp 1965). The amount of radial canals and tentacles, and the shape of the bases of the marginal tentacles are of great importance and the key to distinguish morphologically between other similar jellyfish belonging to the genus *Aequorea*, for example *Aequorea pensilis* and *Aequorea macrodactyla* that bear a close resemblance one to another. The marginal bulb should be determined in order to distinguish between similar species of *Aequorea* (Mayer 1910; Kramp 1956; Kramp 1965; Russell 1970; Bouillon et al. 2006; Gul and Gravili 2013). In our findings we can see clearly (Fig. 1C) the cross-shaped bases of

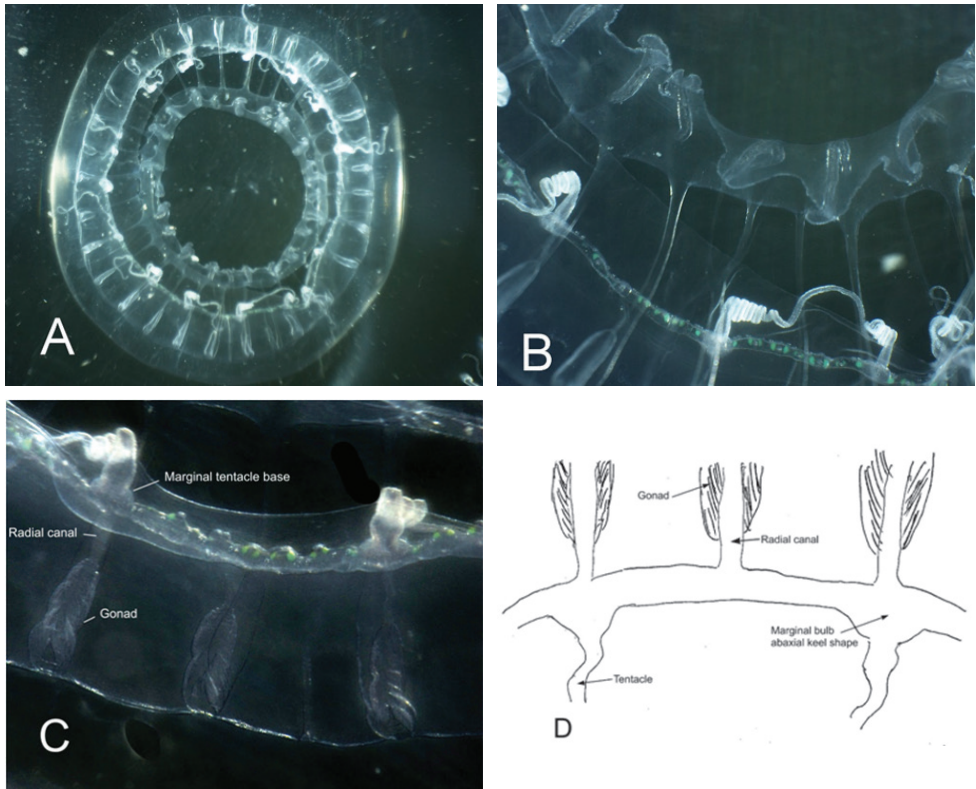


Figure 1. **A** Oral view of the first *Aequorea macrodactyla* collected at the Israeli shoreline in Sdot Yam, on 16.6.2013, size 8 cm **B** Magnification of the rudimentary marginal bulb and the GFP granules **C** The gonads around the radial canal and the shape of the marginal tentacle base **D** A schematic drawing of the marginal bulb tentacle base of the Israeli *A. macrodactyla*.

the marginal tentacles that continue the radial canal. Also it is evident (Fig. 1C) that some radial canals end as marginal bulbs but did not develop tentacles and should be considered as non-tentacular marginal bulbs or rudimentary bulbs (Fig. 1D), which is a characteristic phenomenon of the *Aequorea macrodactyla*. In Figure 1C we can see the position of the gonads surrounding the radial canal. The color of the radial canal, the endoderm of the lips and the tentacle bulbs present a milky color as other parts are mostly transparent.

Genetic identification

The DNA sequences were submitted (Table 1) to the European Nucleotide Archive (ENA).

Our results from the sequencing and processing of different genes through DNA alignment within the population of the Israeli *Aequorea macrodactyla* specimens show very minor differences. These differences are suggested to be negligible and the results

Table 1. Data of *Aequorea macrodactyla* nucleotide Sequence Database, submission to the European Nucleotide Archive.

Eukaryota; Metazoa; Cnidaria; Hydrozoa; Hydroida; Leptomedusae; Aequoreidae; <i>Aequorea</i>			
Accession#: HG964642.	16-JUN-13	<i>Aequorea macrodactyla</i> , partial 16S rRNA gene (Fig. 3)	
Latitude = 32°29'N, Longitude = 34°52'E	Israel. East Mediterranean coast. Sdot Yam		
Primer forward name	16S F756	Forward sequence	CCGTGATAAAGTAGCATAATCAC
Primer reverse name	16S R755	Reverse sequence	AATATTACCCTGTTATCCCTACGG
Accession#: HG964638.	16-JUN-13	<i>Aequorea macrodactyla</i> partial 28S rRNA gene (Fig. 4)	
Latitude = 32°29'N, Longitude = 34°52'E	Israel. East Mediterranean coast. Sdot Yam		
Primer forward name	28S F834	Forward sequence	GAGACCGATAGCGAACAAGTACCGTG
Primer reverse name	28S R833	Reverse sequence	AGAGTTTCCTCTGGCTTCACCCTACTC
Latitude = 32°52'N, longitude = 35°01'E	Israel. East Mediterranean coast. Haifa bay		
Primer forward name	COI F754	Forward sequence	TATGATTATAMGAYTGGAACATATCAGG
Primer reverse name	COI R755	Reverse sequence	GTYAACACATGGTWATYGCCCCAGC

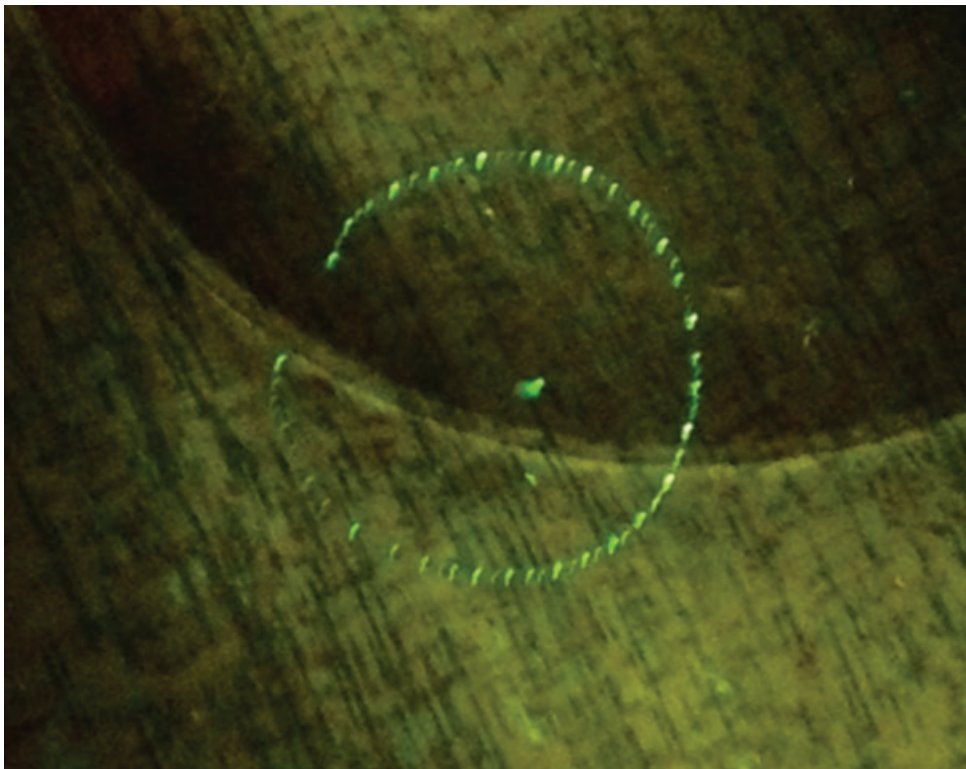


Figure 2. Localization of the Green fluorescent protein (GFP) in the margin of the umbrella in the Israeli *Aequorea macrodactyla*, size 9 cm. Photograph taken inside the boat in Haifa Bay with specimen in sea water using UV illumination with UV flashlight in a darkened room.

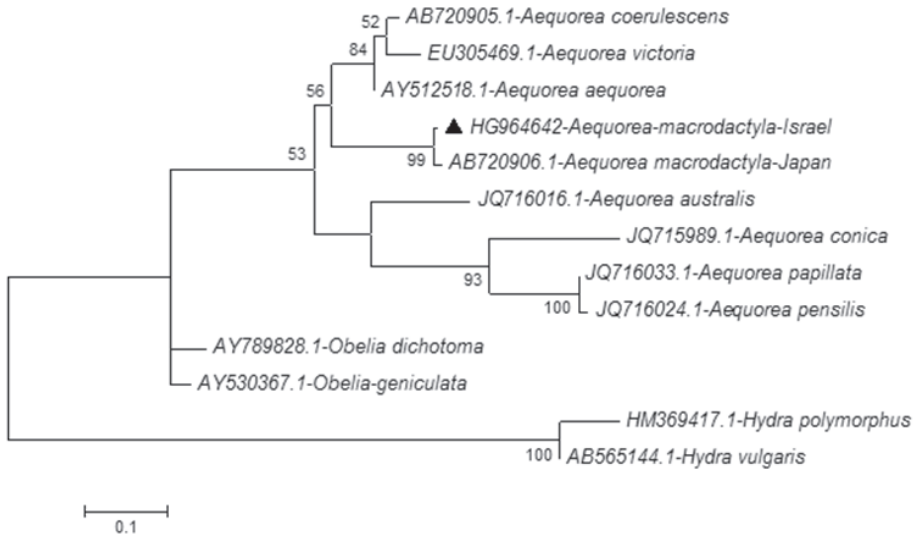


Figure 3. Evolutionary tree for Israeli *Aequorea macrodactyla* by mtDNA 16S marker compared with other members of *Aequorea*. Molecular phylogenetic analysis by Maximum Likelihood Method, an evolutionary tree based on partial sequences of 354 base pairs (bp) mitochondrial DNA (mtDNA) marker 16S. The black triangle represents the local *A. macrodactyla* jellyfish of the Mediterranean according to the DNA molecular sequence acquired in this work. The outgroup for this tree is represented by *Hydra polymorphus* and *H. vulgaris*. The test of phylogeny is by Bootstrap method and the number of bootstrap replications is 1000. For the taxon name we used the NCBI GenBank reference number.

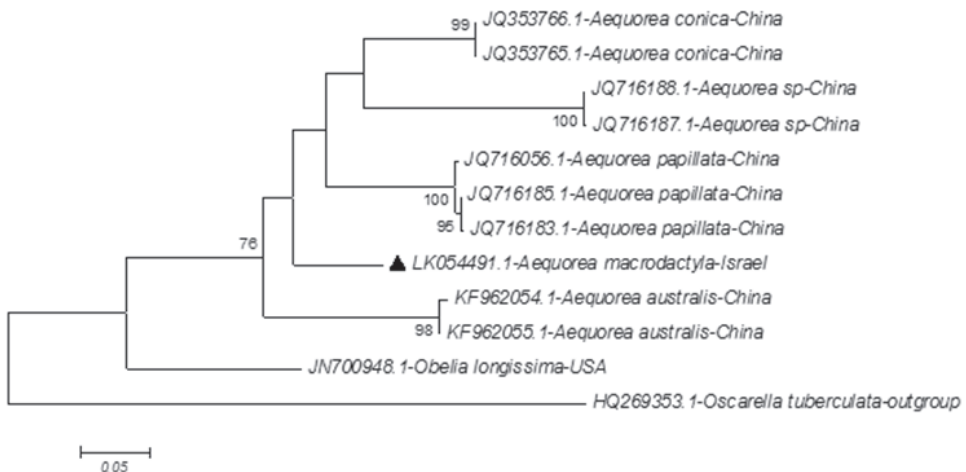


Figure 4. Evolutionary tree by COI marker for Israeli *Aequorea macrodactyla* (black triangle).

show that the Israeli *A. macrodactyla* specimens belong to one group as presented in the following phylogenetic trees (Figs 3, 4, 5) under the reference number HG964642,

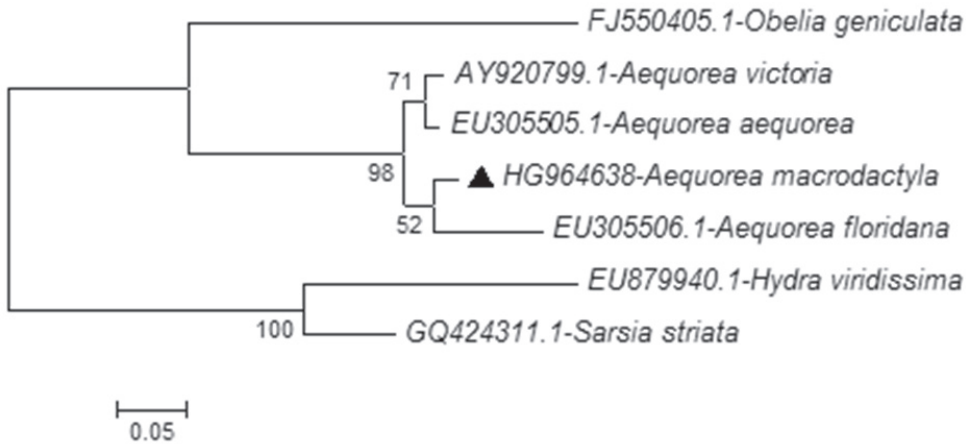


Figure 5. An evolutionary tree of Israeli *Aequorea macrodactyla* by 28S rDNA marker based on specimens from Sdot Yam and Haifa bay (marked by black triangle). The tree is based on partial sequences of 679 base pairs (bp) of the nuclear Ribosomal DNA 28S (rDNA) gene. The outgroup is based on *Hydra viridissima* Pallas, 1766. The reference number used in this figure represents the NCBI GenBank reference number and is followed by the taxon name. The data in this phylogenetic tree include all published data from the species of *Aequorea* existing in NCBI GenBank. The test of phylogeny is by Bootstrap method and the number of bootstrap replications is 500.

which is the National Center for Biotechnology Information (NCBI) GenBank reference number as received from the European Nucleotide Archive (ENA).

The evolutionary history was inferred by using the Maximum Likelihood Method based on the Tamura 3-parameter model (Tamura 1992). The tree with the highest log likelihood (-1506.2803) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree for the heuristic search was obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3008)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 350 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

The tree is based on partial sequences of 691 base pairs (bp) mitochondrial cytochrome c oxidase subunit I (COI) with other family members from the same class. The outgroup is the Porifera, *Oscarella tuberculata*. The test of phylogeny is by Bootstrap method and the number of bootstrap replications is 1000. The reference number used in this figure represents the NCBI GenBank reference number followed by the taxon name.

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Tamura 1992). The tree with the highest log likelihood (-2473.0463) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4266)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated; there was a total of 618 positions in the final dataset (Tamura et al. 2013).

All tree model parameters were estimated by RAxML GAMMA model of rate heterogeneity, ML estimates of alpha-parameter GAMMA Model parameters were estimated up to an accuracy of 0.1 log Likelihood units, Alignment Patterns = 188, DataType = DNA, Substitution Matrix = GTR, and alpha = 0.300374 (Silvestro and Michalak 2012).

Discussion

The Mediterranean Sea is undergoing a dramatic change in biota as a result of biogeographical alterations and ecological changes (Monegatti and Raffi 2001; Monegatti and Raffi 2010; Zenetos et al. 2010; Vermeij 2012), and the East Mediterranean experiences even more changes in biota since the opening of the Suez Canal and fast urbanization along the Israeli Mediterranean coast (Galil et al. 2013). To better understand this ongoing process, it is essential to be familiar with the existing gelatinous fauna in the Mediterranean Sea and to document newly discovered jellyfish and existing ones, so that this important information can be utilized and provide us with the possibility to improve the management of our contributions to the changes in the sea. In this work we utilize phylogenetic and taxonomic relationships with similar Cnidaria to examine if the newly recorded species of *Aequorea* is an immigrant and if so, what constitutes the direction of its invasive pathways and speed of migration (migration vector), or if this species is local (Zenetos et al. 2012; Katsanevakis et al. 2014).

For the species identification of the Israeli *Aequorea*, two phylogenetic systems were combined: traditional morphological phylogenetics and molecular phylogenetic tools. Morphology: shape of the umbrella, position of the gonads, number of radial canals, and the morphology of the marginal bulbs show that the newly found Israeli hydrozoan belongs to the genus of *Aequorea* and we were able to identify this species as *Aequorea macrodactyla* according to the above described characteristics with some minor differences. The identification was made according to the description of this species from other sources (Mayer 1910; Kramp 1956; Kramp 1965; Russell 1970; Bouillon et al. 2006; Gul and Gravili 2013) and the genetic results, which will be discussed further on, support our identification (Fig. 3).

In order to establish the evolutionary history and molecular phylogenetics for the Israeli *Aequorea* widely used DNA markers were employed as a method to identify the species and to construct an evolutionary tree. We used the mitochondrial DNA

(mtDNA) marker 16S (Fig. 3), which is a widely used DNA marker for Hydrozoa; this marker has the most widely available data in the GenBank (Laakmann and Holst 2014). The result, based on partial sequences of 354 base pairs, presented in Fig. 3, supports the morphological results that *Aequorea* of the East Mediterranean Sea appears to be *Aequorea macrodactyla*. The evolutionary tree (Fig. 3) presents all *Aequorea* species by 16S gen available in the NCBI GenBank (Geer et al. 2010) and it is clear that the Israeli and Japanese *Aequorea macrodactyla* are related and different from all other *Aequorea* species presented in this tree (Fig. 3). Furthermore there is a difference in identity of 1% between the two *Aequorea macrodactyla* as shown by the results of 16S nucleotide, using basic local alignment search tool (blast). The Israeli *Aequorea macrodactyla* population shows negligible differences inside the four locally collected groups. 16S mtDNA gene exhibits a very low rate of mitochondrial nucleotide substitution, especially in Cnidaria, where it exhibits a considerably lower rate than in other invertebrates. The rate of substitution for 16S mtDNA in Cnidaria is 10–20 times lower than in vertebrates (Govindarajan et al. 2005). The 1% identity difference between the Israeli and the Japanese *A. macrodactyla* can be an indication of a significant difference between these two *A. macrodactyla* groups, but more worldwide data is required to resolve this issue.

The evolutionary tree resulting from mitochondrial cytochrome c oxidase subunit I (COI) for the Israeli *Aequorea macrodactyla* based on partial sequences of 691 base pairs (Fig. 4) and the evolutionary tree resulting from nuclear Ribosomal DNA 28S (rDNA) gene based on partial sequences of 679 base pairs (Fig. 5) strengthen the previous results from the 16S gene (Fig. 3) for the evolutionary locations of *Aequorea macrodactyla* among other existing *Aequorea* species. In this publication, we provide available sequences for COI and 28S genes and supply a basis for future reference regarding the use of COI and 28S gene markers.

This work emphasizes the importance of using a combination of the two phylogenetic systems especially when one tries to identify fragile and delicate creatures like members of the *Aequorea* spp. Our results show an average of 32 radial canals in the Israeli *A. macrodactyla*. Mayer (1910) wrote that there are 60 to 100 or more radial canals, Kramp (1965) stated that the “numbers of radial canals and tentacles are of great importance” but he presents average numbers of radial canals from 31 to 102, which is closest to our findings. However Kramp (1956) determines that the *A. macrodactyla* contains 153 radial canals. Russell (1970) presents numbers of 60 to 100, even up to 150 radial canals. This example of different and confusing morphological data demonstrates the importance of using both a phylogenetic approach and a genetic approach.

The dynamic changes in gelatinous fauna in the Mediterranean and worldwide are of great interest, as jellyfish play an important role in the stability of marine ecology and the marine food web. In spite of the fact that they are considered simple creatures they rank high in the food web (Mills 2001). Jellyfish can affect the structure of pelagic ecosystems by competing for the same food resource or by predation (Richardson et al. 2009). The current trend, related to global change, reveals a considerable change in the marine biodiversity and marine habitats, reaching the point where marine habitats are experiencing hazardous pressure (Plan 2010). The discovery of *Aequorea macrodactyla*

in the eastern Mediterranean Sea could be interpreted as part of the change in marine biota resulting from the cumulative effects of anthropogenic and global changes that affect the eastern Mediterranean basin. Finding a new species could be explained by the sudden proliferation of a rare indigenous species that up until that point was evasive and stayed out of sight. It is possible that this species flourished as a consequence of changes in the environment that created more favorable conditions. The other possibility, which is more widely accepted, is that they are new immigrants introduced from the Atlantic or the Indo-Pacific, or are Indo-Pacific immigrants that arrived through the Suez Canal as Lessepsian immigrants (Plan 2010).

To deal with these important questions, whether *Aequorea macrodactyla* has been an evasive hydromedusa that kept a low profile or whether this is a new invasion by a non-indigenous species, we examined three DNA markers (Table 1): mitochondrial DNA (mtDNA) marker 16S (Fig. 3), mitochondrial cytochrome c oxidase subunit I (COI) marker (Fig. 4), and Ribosomal DNA 28S (rDNA) genes (Fig. 5). The results from marker 16S (Fig. 3) suggest that the genetic differences between the Israeli and the Japanese population are small but existent, and can be interpreted as a normal divergence within a population. As the Israeli *A. macrodactyla* population was collected from different places and at different times and demonstrated very little genetic divergence without any distinctive variance at all, we suggest that the local *A. macrodactyla* are not native to the East Mediterranean and should be considered a new immigrant jellyfish; we propose that the jellyfish from the Far East is the source of the Israeli East Mediterranean *Aequorea macrodactyla*.

The arrival of *A. macrodactyla* to the East Mediterranean could be explained by one of two possibilities, either Lessepsian migration or human transportation. Lessepsian migration is not a well-defined term and we propose to define it as successful migration of marine creatures from one side of the Suez Canal to the other, coming through the Suez Canal either step by step, swimming or even using local transportation (Por 1971) as long as the Canal enabled this immigration. For example, a hydromedusa arriving from the Arabian Sea to the Mediterranean by ballast water cannot be considered a Lessepsian migrant but should be defined as an anthropogenic migrant by means of human transportation. Human transportation is a means for the marine creature to immigrate to a new location, regardless of the transportation route: at present this appears to be the main path of introduction of a species (Gravili et al. 2013). As we compare our *A. macrodactyla* with the existing data one can assume that either one of the immigration options presented here is possible.

For *A. macrodactyla* in the seas adjoining the Mediterranean, records exist from the Bay of Eilat, the Gulf of Aden, the Red Sea, and the English Channel. All these published records are old i.e. from more than four decades ago, and the nearest and newest reports come from the Indian Ocean (23 July 2007) and from the North Atlantic in Central America on 15 September 1977 (Encyclopedia of Life 2014). The report of *A. macrodactyla* in the Red Sea (Schmidt 1973) is old, more than 40 years ago, and there have been no further records of *A. macrodactyla*. Moreover, Gravili et al. (2013) proposed to remove species from the list of taxonomic records that have not been found for a reasonable time (several decades), and we concur.

To summarize this discussion, we suggest that this jellyfish is an immigrant that used ship transportation from the Indo-Pacific to the Mediterranean, which is supported by the high DNA similarity with the Japanese (Xia et al. 2002) *Aequorea macrodactyla* (Fig. 3) and by the lack of other evidence or reliable information for this jellyfish existing in the Red Sea in recent years.

In this paper we emphasize the dynamic process in the East Mediterranean marine fauna as a significant part of the ecosystem's change in natural balance and as a result the introduction of a new species and change in the biodiversity. Moreover, genetic tools were utilized as a means for distinguishing and examining newly recorded species to determine whether this is a native species or an immigrant. As we pointed out, global information is limited, more work should be carried out, especially in the East Mediterranean as the marine habitat and its fauna are changing rapidly. The results indicating Indo-Pacific migration could be interpreted as part of the change in marine biota as a result from the cumulative effects of anthropogenic and global changes that affect the eastern Mediterranean basin (Duarte 2014; Tsikliras and Stergiou 2014).

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