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A complex invasion story underlies the fast spread of the invasive box tree moth (*Cydalima perspectalis*) across Europe

[1]Audrey Bras, [2]Dimitrios N. Avtzis, [3]Marc Kenis, [4]Hongmei Li, [5]Gábor Vétek, [1]Alexis Bernard, [1]Claudine Courtin, [1]Jérôme Rousselet, [1]Alain Roques, [1]Marie-Anne Auger-Rozenberg

[1]INRA, UR633 Unité de Recherche de Zoologie Forestière, 2163 Avenue de la Pomme de Pin, CS 40001 ARDON 45075 ORLEANS Cedex 2, France

[2]Forest Research Institute, Hellenic Agricultural Organization Demeter, Vassilika, Thessaloniki, Greece

[3]CABI, Delémont, Switzerland

[4]MoA-CABI Joint Laboratory for Biosafety, Institute of Plant Protection, Chinese Academy of Agriculture Sciences, Beijing, China

[5]Department of Entomology, Faculty of Horticultural Science, Szent István University, Villányi út 29–43, H-1118 Budapest, Hungary

Corresponding author: Audrey Bras, tel: +33 2 38 41 80 21, audreyb061992@gmail.com

Abstract

Many recently established non-native insect species appear to be spreading across Europe significantly faster than before. The box tree moth (*Cydalima perspectalis*), a native to Asia, is illustrative of this trend. First recorded in 2007 in Germany, the moth has then colonized in less than 10 years more than 30 countries in Europe and Asia Minor, causing significant damage to wild and ornamental *Buxus* trees. It has been hypothesized that the trade of ornamental box trees between China and Europe was responsible for the moth introduction while plant trade among European countries may have caused its rapid spread. To clarify the pest invasion history, we analyzed the genetic diversity and structure of its populations in the native and invaded ranges, using a 1495-bp fragment of the mitochondrial cytochrome oxidase I and II genes. Moth genetic diversity in Asia compared to the one observed in the invaded Europe and Asia Minor suggested that the invasive populations probably originated from eastern China. Furthermore, the high genetic diversity coupled with the spatial genetic structure in the invaded range suggested the occurrence of several introduction events, probably directly from China. Moreover, the spatial genetic structure in Europe and Asia Minor may also reflect secondary invasions within invaded range because of ornamental plant trade among European countries.

Keywords: *Cydalima perspectalis*, *Buxus*, invasion, insect, ornamental plant trade, multiple introductions

Key Message:

The invasive moth, *Cydalima perspectalis*, has spread rapidly across Europe and Asia Minor, causing significant damage to both wild and ornamental *Buxus* trees.

Genetic analyses suggested China, and mainly eastern China, as the source of the populations invasive in Europe but also that multiple introduction events likely occurred.

The fast spread of the moth across Europe may result from a combination between these multiple introductions and human-mediated long-distance transportations of infested *Buxus* trees with ornamental plant trade among European countries.

Author Contribution Statement

ABr, JR, AR, MAAR conceived the research. ABr, DA, MK, HM, GV, ABe, AR performed sampling. ABr, DA, CC performed experiments. ABr, DA analyzed data. ABr, DA, GV, JR, AR, MAAR wrote the paper. All authors approved the paper.

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Introduction

Biological invasions are continuing to occur worldwide despite an increasing awareness of how globalization is resulting in human-mediated introductions of species with major ecological, economic, and sociological effects (Roques 2010a; Simberloff et al. 2013; Meurisse et al. 2018). Moreover, the arrival of new non-native species is not showing any signs of plateauing (Seebens et al. 2017). In Europe, most of the insect species that have arrived during the recent decades are first-time invaders, which have never been categorized as invasive elsewhere (Seebens et al. 2018). Most of newly insect invaders are originating from Asia (Roques 2010a), with an ever-increasing proportion of phytophagous species (Roques et al. 2016). The ornamental plant trade is considered as the major pathway by which these phytophagous insects have been accidentally introduced into Europe (Kenis et al. 2007; Roques 2010a; Eschen et al. 2017), in parallel with a significant increase in the importation of live plants to the continent since 1995 (Van Kleunen et al. 2018). Quite simultaneously, faster rates of spread following establishment were observed in many of these recently-introduced insects (Roques et al. 2016). Unregulated trade, especially of ornamental plants, as well as the progressive liberalization of trade and travel during the 1990s may have facilitated such rapid expansions of non-native species across Europe (Roques et al. 2016).

The box tree moth, *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae), a native to Asia, is representative of the non-native species having spread rapidly across Europe. Its native range includes China, Korea, and Japan (Maruyama and Shinkaji 1987; Xiao et al. 2011; Kim and Park 2013), where the insect is known to develop on several *Buxus* species (Buxaceae) (Wan et al. 2014). In Europe, the moth larvae only feed on leaves and shoots of *Buxus* species (Leuthardt and Baur 2013; Matošević et al. 2017), eventually causing plant death (Kenis et al. 2013; Wan et al. 2014). *C. perspectalis* was first observed on ornamental box trees in urban areas but it has now spread to natural forests in some countries, causing severe defoliation in native box stands, such as for *B. sempervirens* and *B. colchica* (Kenis et al. 2013; John and Schumacher 2013; Gninenko et al. 2014; Mitchell et al. 2018). *C. perspectalis* was first recorded in Europe in early 2007, at two different sites in Germany (Krüger 2008). Later that same year, it was observed in Switzerland and the Netherlands (Leuthardt et al. 2010; Van der Straten and Muus 2010) (Figure 1). Then, over a period of less than 10 years, the insect spread across the whole of Europe and into Asia Minor, and it is at present observed in more than 30 countries, ranging from the United Kingdom (Salisbury et al. 2012) to Iran (Mitchell et al. 2018).

It has been hypothesized that the moth was accidently introduced primarily via the trade of ornamental box trees between China and Europe (Leuthardt et al. 2010; Casteels et al. 2011; Nacambo et al. 2014), and that subsequent trade among European countries led to its fast spread (EPPO 2012; Kenis et al. 2013; Matošević 2013). Indeed, China shipped large quantities of *Buxus* trees to several European countries between 2006 and 2010 (EPPO 2012). As a result, the moth could have been introduced several times in different countries after its initial appearance in 2007 in Germany. However, few interception records are available from the European plant health and quarantine services (EPPO 2012) because the moth was included on the EPPO alert list only from 2007 to 2011 (Strachinis et al. 2015). During this period, *C. perspectalis* was intercepted once in 2008 in the Netherlands (EPPO 2012), a country that has been the largest importer of ornamental plants to Europe over the recent years (Eschen et al. 2017). It is also noticeable that in the Netherlands, Belgium, and England, the moth was first recorded from nurseries (Van der Straten and Muus 2010; Casteels et al. 2011; Salisbury et al. 2012) whilst the first mention in Russia was on box trees imported for the Winter Olympics in Sochi (Gninenko et al. 2014). Actually, box trees, especially *Buxus sempervirens*, are very popular ornamental plants (Matošević 2013; Mitchell et al. 2018) and drive significant commercial trade within Europe and adjacent countries (EPPO 2012; Dehnen-Schmutz et al. 2010).

Human-mediated introductions often involve complex invasion pathways (Garnas et al. 2016; Meurisse et al. 2018), for which historical information may be missing or misleading. Hence, molecular data can often supplement what is known about a species' invasion history, helping to clarify the likely invasive pathways by revealing the presence of e.g. genetic bottlenecks, multiple introduction events, or admixture (Estoup and Guillemaud 2010; Lawson Handley et al. 2011; Estoup et al., 2016; Fraimout et al. 2017). The sequencing of mitochondrial DNA (mtDNA) is an efficient first step for disentangling the pathways followed by non-native species. It can be used to identify source populations, founding events, and the occurrence of multiple introductions (Muirhead et al. 2008; Estoup and Guillemaud 2010; Cristescu 2015). Thus, this approach has been employed in a number of non-native species (e.g., Auger-Rozenberg et al. 2012; Gariepy et al. 2014; Javal et al. 2017; Lesieur et al. 2018). Identifying source populations and clarifying invasive pathways help to complement management strategies and increases our understanding of how non-native species spread (Muirhead et al. 2008; Lawson Handley et al. 2011).

The objectives of this study were to clarify the geographical origin(s) and colonization history of the populations having invaded Europe. To this end, we compared the genetic diversity and structure of the populations of the box tree moth in the native Asian range and in most of the invaded countries of Europe and Asia Minor, using mtDNA cytochrome oxidase I and II (COI - COII) genes.

Materials and methods

Sampling

Moth populations were sampled throughout their native and invaded ranges (Table 1; Supplementary Materials Table S1, Figures S1 and S2).

In the native Asian range, we attempted to collect specimens from 2012 to 2017 in both natural stands and ornamental plantations of *Buxus* trees growing in the putative distribution range of the moth (Figure 2a). Pheromone traps were used for adult moths and hand sampling for larvae and pupae (Suppl. Mat. Table S1). However, surveys and trappings were unsuccessful in Japan even though *C. perspectalis* has been recorded there (Maruyama and Shinkaji 1987; Kawazu et al. 2007). In South Korea, we sampled one population from an urban area in Seoul (Table 1; N20). In China, 19 populations were obtained, covering most of the putative range from north-eastern China (Liaoning province), northern China (Nei Mongol and Beijing provinces), and eastern China (Shandong, Anhui, Jiangsu, Shanghai, Zhejiang, and Fujian provinces), to south-central China (Henan province) and south-western

China (Yunnan and Guizhou provinces). Only the southern provinces of Guangdong and Guangxi could not be sampled. However, all but one of these Chinese populations were collected in urban areas despite our efforts in natural stands of *Buxus* where moth density appeared to be very low. Only in Fuyang, Zhejiang province (Table 1; N17), larvae could be collected outside urban areas, in a sentinel plant nursery established at the boundary between a natural forest and agricultural lands (Kenis et al. 2018).

In the invaded range, samples could be collected in 23 different countries (Table 1) from regions that were colonized by the moth between 2007 and 2016 (Figures 1 and S2). Ideally, we intended to collect larvae via hand sampling. When that was not possible, adults were captured using pheromone traps. Moths were mostly collected in urban areas except in the following sites: Bzyb Valley (Table 1; I27) and Mtirala Park in Georgia (Table 1; I28); all sites in Russia (Table 1; I58-I60); Si Sangan National Park in Iran (Table 1; I41); and Roquefort-sur-Garonne (Table 1; I24) and Marcillac-Vallon in France (Table 1; I23).

Upon collection, all specimens were placed in 96% alcohol and stored at -21°C to preserve their DNA until the analyses could take place.

DNA extraction, amplification, and sequencing

The larvae and adults were dissected. DNA was extracted from the thoracic muscles of the larvae or the legs of the adults using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany). We amplified a section of the mitochondrial genome that included part of the COI-COII genes. We employed a pair of primers developed for a related species, *Diaphania* (=*Glyphodes*) *pyloalis* (Zhu et al. 2013). The primers were renamed LeCyd-F2 (5’ TGGAGCAGGAACAGGATGAAC 3’) and Cynna-R2 (5’ GAGACCANTACTTGCTTTCAG 3’). Amplification was carried out in a total PCR volume of 25 µL, which contained 1 µL of DNA, 15.8 µL of ultrapure water, 2.5 µL of 10X DreamTaq Green Buffer, 2.5 µL of dNTP (10mM), 0.5 µL of $MgCl^2$ (2.5mM), 1 µL of each primer (10µM), 0.5 µL of betaine solution (5 M), and 0.2 µL of DreamTaq DNA polymerase (5 units/µL). Thermocycling was performed using a Veriti® 96-well Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and the following procedure: an initial 5-min denaturation step took place at 95°C and was followed by 25 amplification cycles (94°C for 35 s, 60°C for 45 s, and 72°C for 3 min). PCR products were analyzed by gel electrophoresis in a 1.5% agarose gel to check for successful amplification. Those of approximately 2000 bp in length were purified using the NucleoFast® 96 PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). A fragment of around 1500 bp of purified DNA that included COI, ARNt L2, and COII was then sequenced using Cynna-R2 and the internal primer Jerry-F 5’ CAACATTTATTTTGATTTTTTGG 3’ because the PCR products were too long to be sequenced directly. Sequencing was carried out using the Big Dye Terminator Cycle Sequencing Kit (v. 3.0, Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3500 Genetic Analyzer. The two sequenced strands were then aligned and the absence of double peaks on electropherograms was manually verified using CodonCode Aligner v. 3.7.1 (CodonCode Corporation, Centerville, MA, USA).

Genetic Analyses

We successfully sequenced mtDNA from 132 and 305 individuals from the native and invaded ranges, respectively. Sequence data from Matošević et al. (2017) was also included in the dataset for the invaded range. Sequences were aligned using Clustal W (Thompson et al. 1994), which was implemented in BioEdit v. 7.1. The final alignment was obtained without any insertions or deletions. All sequences were truncated at the same length (1495 bp). The presence of stop codons were checked using MEGA v. 6 (Kumar et al. 2008). We compared our sequences with sequences in GenBank and BOLD using BLAST to confirm that individuals had been properly identified as *C. perspectalis* and to check for possible contamination.

A phylogenetic analysis to investigate the relationships among mtDNA haplotypes was performed by maximum-likelihood (ML) method computed using the software MEGA v. 6. Evaluation of statistical confidence in nodes was based on 10,000 boostrap replicates. The distance between DNA sequences were calculated based on Kimura's two-parameter method (Kimura 1980). Two related species, *D. pyloalis* (Genbank Accession No. KM576860) and *Glyphodes quadrimaculalis* (Genbank Accession No. KF234079), were used as outgroup taxa. A statistical parsimony network with a 95% confidence level was constructed using TCS v. 1.21 (Clement et al. 2000). We determined haplotype number (H), haplotype diversity (*h*), the average number of nucleotide differences (k), and nucleotide diversity (*n*) using DNAsp v. 5 (Librado and Rozas 2009). Haplotype distribution and frequency were projected onto maps of Asia, Europe, and Asia Minor using ArcGis v. 10.6 (ESRI, Redlands, CA, USA). To characterize the moth's native range, spatial genetic structure was first assessed by testing if G_{ST} (the coefficient of genetic variation over all the populations) was significantly smaller than N_{ST} (the coefficient taking into account similarities among haplotypes)—10,000 permutations were implemented in Permut (Pons and Petit 1996).

Analysis of molecular variance (AMOVA) was performed to look for evidence of genetic structure in the moth's native and invaded ranges using Arlequin v. 3.5 (Excoffier and Lischer 2010). To carry out the analysis, we grouped the populations according to different criteria. First, we took into account the populations' geographical locations. Second, we took into account information related to the ornamental plant trade, such as production areas and volume of imported and/or exported plants. The objective was to estimate the impacts on genetic structure in both the native and invaded ranges simultaneously. In the native range, sequences were grouped into five clusters based on population geographical location and known *Buxus* tree production in Chinese provinces (René Eschen pers. comm.) (Figures 2a and S1): (1) north-eastern province of Liaoning grouped with South Korea (N8, N20); (2) northern province of Nei Mongol and Beijing area (N2, N9); (3) eastern provinces of Fujian, Henan, Shandong, and Shanghai area (N3, N4, N6, N10, N11, N12, N13, N14); (4) south-eastern provinces of Anhui, Jiangsu and Zhejiang (N1, N7, N17, N18, N19); and (5) south-western provinces of Guizhou and Yunnan (N5, N15, N16). In the invaded range, information on ornamentals was country-specific (Dehnen-Schmutz et al. 2010; EPPO, 2012; Eschen et al. 2017), and therefore sequence data were first grouped per country. Then, countries were grouped into four clusters based on geographical location and the commercial value of ornamental plant imports/exports (Dehnen-Schmutz et al. 2010; Eschen et al. 2017). The groups were as follows: (1) Germany, Netherlands, France, Italy, Belgium; (2) Switzerland, Spain, Portugal, Luxembourg; (3) Austria, Hungary, Czech Republic, Slovenia, Slovakia, Croatia, Greece, Serbia; and (4) Romania, Turkey, Russia, Bulgaria, Georgia, Iran.

Results

Twelve haplotypes were identified based on the sequences of the 437 specimens collected across the species' native and invaded ranges (HTA1-HTC1, Table 1; GenBank accession numbers: MK611945-MK611956). These haplotypes comprised 21 single nucleotide polymorphisms. There was no evidence of contamination nor of nuclear copies of mitochondrial DNA (numts). The BLAST search confirmed specimen identification, based on the few samples of *C. perspectalis* present in GenBank and BOLD.

The haplotypes formed three haplogroups (A, B, and C), which were separated by at least seven mutation steps (Figure 2b). The sixteen intermediate haplotypes were not present in our sample pool. The topology of the phylogenetic tree was similar to the haplotype network (Suppl. Mat. Figures S3). Two haplotypes, HTA1 and HTB1, were prevalent—they were displayed by approximatively 36**%** of the individuals sequenced. Two other haplotypes, HTA2 and HTB2, had prevalence of 13.5% and 8.9%, respectively. These four haplotypes were found in both the native and invaded range. Overall, haplotype diversity (*h*) was 0.709 (± 0.012), the average number of nucleotide differences (k) was 4.10, and nucleotide diversity (*n*) was 0.00274 (± 0.0000).

Genetic diversity and structure in the native range

Twelve haplotypes were observed among the sequences of the 132 specimens collected in China (19 sampling locations) and South Korea (1 sampling location) (Table 1, Figures 2a and 2b). The average pairwise sequence difference between haplotypes was 0.004, and ranged from 0.1% to 0.7%, which is consistent with intraspecific distances. The three haplogroups were represented. Haplogroups A and B were observed across the range of Chinese populations, but haplogroup C was only found in the Fuyang population (N17, Zhejiang province). Haplogroups A and B comprised 5 and 6 haplotypes, respectively, while haplogroup C was made up of only one. In most populations (65%), haplotypes belonging to haplogroups A and B co-occurred (e.g., Beijing, N2; Ordos, N9; Tai'an, N11). The values of the diversity indices are provided in Table 1.

Only haplotype HTB1 was common to both South Korea and China (Figure 2a), where it was shared by 32.6% of the individuals sequenced. HTB3 was only observed in South Korea (N20: 46%). HTA1 and HTB2 were prevalent across China (30.3% and 22.0%, respectively). HTA1, HTB1, and HTB2 occurred in 14 populations. With the exception of HTA2 and HTA4, which were each found in two populations in different provinces (HTA2: N9, Nei Mongol province, and N11, Shandong province; HTA4: N12, Shandong province, and N14, Shanghai province), all the other haplotypes were observed at single locations in China. Nine populations contained three haplotypes, whereas three populations contained a single haplotype. Among these three populations, only in the Wendeng population (N13, Shandong province) was more than one individual sampled. In native populations, haplotype number (H) ranged from 1 to 4, haplotype diversity (h) ranged from 0 to 1, the average number of nucleotide differences (k) ranged from 0.40 to 5.98, and nucleotide diversity (n) ranged from 0 to 0.004.

Pronounced genetic structure was observed: N_{ST} (0.277) was significantly higher than G_{ST} (0.181; p value < 0.01). The AMOVA results also supported the existence of genetic structure as all the fixation indices were significant (p value < 0.05), including F_{CT} (Table 2). Genetic differences among groups and among populations within groups nevertheless accounted for a small percentage of the genetic variance (8.4% and 9.4% respectively). The largest amount of genetic differentiation (82.2%) was found within populations (highly significant fixation index; p value < 0.001).

Genetic diversity and structure in the invaded range

Only five haplotypes (HTA1, HTB1, HTA2, HTA4, HTB2) emerged from the sequences of the 305 specimens obtained from the 72 locations sampled in Europe and Asia Minor. All of these haplotypes were also present in China (Table 1). The values of the diversity indices in the invaded range were lower than those in the native range (h= 0.668, k= 3.79, and n= 0.0027).

HTA1 and HTB1 were observed across the invasive populations (Figure 3a and 3b). HTA1 was found in 47 populations and 39.3% of the individuals sequenced, while HTB1 was found in 45 populations and 38.0**%** of the individuals sequenced. HTA2 was seen in 29 populations. HTA4 and HTB2 had restricted distributions—they occurred in populations associated with the first records of *C. perspectalis* in Europe. HTA4 was only found in the Weil-am-Rhein population (I34) in Germany and in the Saint Louis population (I18) in France, which is located near the border with German. HTB2 was found mostly in populations in Kehl (I33) and Strasbourg (I19), two nearby locations. Twenty-eight populations contained just one haplotype, whereas 34 populations contained two haplotypes, and 9 populations contained 3 haplotypes. In invasive populations, haplotype number (H) ranged from 1 to 4, haplotype diversity (h) ranged from 0 to 1, the average number of nucleotide differences (k) ranged from 0.4 to 8, and nucleotide diversity (n) ranged from 0 to 0.00535. We did not observe any genetic diversity in Switzerland, Austria, and Greece. Germany and France had the highest genetic diversity: four and five haplotypes, respectively.

The AMOVA results revealed the presence of genetic structure within the invasive populations (Table 2). The values of all the fixation indices were highly significant (p value < 0.01). The largest amount of genetic differentiation (76.09%) was found within populations. Genetic differences among populations within groups and among groups accounted for a smaller percentage of genetic variation (12.30% and 11.61%, respectively).

Discussion

Based on mtDNA sequence diversity, three major findings resulted from this study. First, the box tree moth displayed a complex genetic structure with a mix of deeply differentiated haplogroups in its native Asian range, probably as a result of anthropogenic activities within this region. Second, the comparison of the genetic diversity patterns between Asia and the invaded Europe indicated eastern

China as the likely source of the European populations. Finally, historical records coupled to the genetic diversity and structure observed in the invaded range suggested that multiple introduction events may have occurred.

Genetic structure of the box tree moth in its native range

Cydalima perspectalis displayed a weak but significant spatial genetic structure across its native range. From our analyses, three main distribution regions can be delimited based on: (1) the occurrence of a private haplotype (HTB3) in South Korea, (2) the co-occurrence of haplogroups A and B in northern and eastern China; and (3) the high prevalence of haplogroup B in southern China. Similar phylogeographic pattern was observed in some lepidopterans native to Asia. The Asiatic rice borer, *Chilo suppressalis*, thus showed three genetically diverse and geographically localized clades in China corresponding to north-eastern, central and southern China (Meng et al. 2008). These same major lineages were also defined for a swallowtail, *Papilio bianor* (Zhu et al. 2011). Actually, these regions have been pointed out as Glacial refugia for the last two species (Meng et al. 2008; Zhu et al. 2011). Since *Buxus* fossils were found in southern China (Ma et al. 2015; Huang et al. 2018), this region may also have served as refugia for some populations of *C. perspectalis*.

Only specimens collected from urban areas could be analyzed because we were unable to obtain samples from natural stands of *Buxus* despite intensive efforts. Indeed, the moth’s putative distribution in Asia appeared to be solely based on records from urban areas (Kawazu et al. 2007; Kim and Park 2013; Nacambo et al. 2014), which makes it difficult to define its natural distribution. A large part of the natural stands of *Buxus* species are found in southern China (Figure 2a) (Fang et al. 2011). Thus, the proximity to forests may explain the significant genetic diversity that we observed in our data, even if our samplings in this region were carried out on box trees planted in towns. Similarly, the proximity of the sentinel plant nursery in Fuyang (south-eastern China) to natural stands may also account for the unique occurrence of haplogroup C at that site.

The mtDNA diversity of *C. perspectalis* populations in Asia corresponds to three distinct haplogroups, two of which being widely distributed. The spatial co-occurrence of divergent haplogroups suggests that this moth has a complex history. Indeed, the existence of divergent haplogroups may reflect ancient phylogenetic differentiation (Avise et al. 1987), whereas their co-occurrence may have resulted from secondary contact, produced by moth movements and/or by human-mediated dispersal of infested plants. Haplotype co-occurrence has also been observed in the native Chinese range of the Asian long-horned beetle, *Anoplophora glabripennis*, leading to suggest that beetles have been moved by man to northern and eastern China with plants used for reforestation (Carter et al. 2009; Javal et al. 2017). The oriental fruit moth, *Grapholita molesta*, displayed the same genetic pattern, which likely reflect recent dispersal through human activities (Song et al. 2018).

More generally, human-mediated dispersal can indeed reshape the genetic structure of insect populations and largely modify their primary natural phylogeographic pattern (Stone et al. 2007; Song et al. 2018). In recent years, *Buxus* trees have increasingly been planted as ornamentals in China, especially in the northern part of the country. Because no box trees grow in the wild in northern China, it can be assumed that *C. perspectalis* has been introduced into the cities of this region (e.g., Beijing) as a result of ornamental plantations (Nacambo et al. 2014). As our samplings were essentially carried out in urban areas, the observed genetic structure is likely to represent a combination between the moth phylogeographic history and its human-mediated dispersal with ornamental plant trade. Additional samplings in natural stands, especially from other provinces of south-central China and Japan would be required to precise the evolutionary history of *C. perspectalis* in Asia and understand better its present spatial genetic structure.

A Chinese origin for the populations invasive in Europe and Asia Minor

Five haplotypes were observed in *C. perspectalis* invaded range. It corresponded to a significant fraction (41.7%) of the genetic variability observed in the native Asian populations. This pattern was commonly noticed in biological invasions (Dlugosch and Parker 2008; Estoup and Guillemaud 2010; Lawson Handley et al. 2011; Cristescu 2015). For example, a high genetic diversity in the invaded range was also observed for the micromoth *Phyllonorycter issikii* (Kirichenko et al. 2017), and the brown marmorated stink bug, *Halyomorpha halys* (Gariepy et al. 2014, 2015), two invaders that came to Europe from Asia.

For *C. perspectalis*, the South Korean haplotype was not observed in the invasive populations whereas all the haplotypes found in the invaded range were also observed in China, including two of the three most common haplotypes. This finding strongly suggested that these invasive populations have a Chinese origin. It could be coherent with the data on *Buxus* tree imports (EPPO 2012), which also pointed out China as the most probable source of invasion (Leuthardt et al. 2010; Casteels et al. 2011; Nacambo et al. 2014). Over recent decades, China has effectively emerged as a key exporter of ornamental plants (Dehnen-Schmutz et al. 2010; Kenis et al. 2018). For example, between 2005 and 2010, the Netherlands obtained more than 80% of its imported ornamental plants (i.e., import volume) from eastern Asia, and especially from China (van Valkenburg et al., 2014). Moreover, China was the greatest supplier of *Buxus* trees to other European countries during that same period (EPPO 2012; Kenis et al. 2018).

Both the spatial genetic structure of the moth populations within China, the distribution of the production areas of ornamental *Buxus* in this country, and the higher economic development of coastal Chinese provinces (Roques 2010b) suggested eastern China as the location of the invasive source. All the haplotypes observed in Europe were also found in the Shandong province, and four were observed around Shanghai. The coastal provinces in eastern China are more economically developed than are other Chinese provinces, and Shanghai is one of China's key economic centers, from whence large quantities of products are exported throughout the world (Roques 2010b; Lu et al. 2018). Moreover, half of the areas in which *Buxus* trees are produced for export are found in eastern China, notably around Shanghai (René Eschen pers. comm.). These places could potentially be the sources of invasive *C. perspectalis* populations. However, correctly identifying source populations or invasion pathways is not always straightforward. The native range of an invader can be too large to be exhaustively characterized (e.g., Orlova-Bienkowskaja et al. 2015; Orlova-Bienkowskaja and Volkovitsh 2018). The fine-scale reconstruction of an invasion history is possible when there is significant genetic structure in the native range (Lombaert et al. 2011; Cristescu 2015). Here, it was challenging to define the source of the invasive moth populations because there was a significant genetic diversity in the invaded range coupled to a low level of genetic variation among the analyzed Chinese populations, and a lack of samples from populations from other native areas in southern China and Japan.

A complex invasion process

The populations sampled where *C. perspectalis* was first recorded in 2007 in Europe (Krüger 2008; Van der Straten and Muus 2010; I34 and I33 in Germany and I52 and I53 in the Netherlands;) differed genetically. German populations presented two haplotypes, which were missing in the Netherlands, whereas the Dutch populations showed one haplotype not found in Germany. Moreover, three of these four populations shared only one haplotype. This pattern could result from (i) a single founder event with a significant genetic diversity introduced before 2007 in one of these localities, followed by a secondary spread. In the invasion processes, it is common knowledge that insects can be present before their first observations (Allendorf and Lundquist 2003; Crooks 2005). Furthermore, this genetic diversity could reflect the introduction of insects belonging to populations from eastern China, in which we observed often signs of admixture. Such a pattern was already observed for *Ambrosia artemisiifolia*, the annual weed which recently invaded Europe (van Boheemen et al. 2017).

The genetic pattern of *C. perspectalis* can also be explained by (ii) multiple introductions arriving directly from China to several places at the same period. *Buxus* trees were commercially imported from China by different countries of the EU, at least the Netherlands and Italy, between 2006 and 2010 (EPPO 2012). No information is available for Germany, but Weil am Rhein where the insect was first observed, regularly received large shipments of Chinese imports (Casteels et al. 2011). This trade information coupled with our genetic data may suggest more than one introduction directly from China. These events of multiple introductions are a common feature in biological invasions (Estoup and Guillemaud 2010; Lawson Handley et al. 2011; Cristescu 2015). It has been observed for many non-native insects across the world (e.g., *Diabrotica virgifera virgifera*, Ciosi et al. 2008; *Hyalopterus pruni*, Lozier et al. 2009; *Cactoblastis cactorum*, Marsico et al. 2011; *Leptoglossus occidentalis,* Lesieur et al. 2018), and recently, for non-native species originating from China (*Harmonia axyridis*, Lombaert et al. 2010; *H. halys*, Gariepy et al. 2015; *A. glabripennis*, Javal et al. 2017; *Drosophila suzukii*, Fraimout et al. 2017).

In the same way, multiple introductions may increase the probability of spread of non-native species inside the invaded range (Cristescu 2015). Indeed, anthropogenic activity is known to promote subsequent introduction events, leading to dispersal and range expansion (Estoup and Guillemaud 2010). It is assumed that *C. perspectalis* has a natural dispersal rate of around 10 km per year (Van der Straten and Muus 2010; Casteels et al. 2011), which is far too limited to explain its fast colonization of Europe and Asia Minor. The spatial genetic structure suggested that different groups exist. This pattern likely resulted from several complex introduction events, some possibly directly from China and/or some within the invaded range. The speed of the invasion can be explained by those different assumptions, even if the important ornamental plant trade inside Europe (Dehnen-Schmutz et al. 2010; Eschen et al. 2015) may have clearly played a role in the moth dispersal. For example, in 2008, the moth was intercepted in the Netherlands in a *Buxus* shipment of unknown provenance for exportation inside Europe (EPPO 2012). Besides, Gninenko et al. (2014) supposed that the insect was introduced from Italy in 2012 in Caucasian forests due to the import of infested box trees for the Winter Olympics in Sochi. If considering Hungary, Turkey and Romania, where the pest was recorded in 2011 (Sáfián and Horváth 2011; Hizal et al. 2012; Gutue et al. 2014), the geographical distances separating the localities are greater than 400 km (Figure 1), which suggests simultaneous introductions but from unknown origins.

Our study provides a new example of a pest species with a complex invasion history. We have highlighted the role played by the ornamental plant trade in its introduction and dispersal, even if more detailed data on *Buxus* tree imports to Europe are needed to decipher the number of introduction events. However, to better pinpoint the moth's region of origin in China and to disentangle the effects of anthropogenic activity on its current distribution patterns, we need to carry out broader sampling efforts and acquire more information about the moth's natural distribution in its native range (Muirhead et al. 2008). Additionally, more powerful tools such as microsatellite or SNP markers (Estoup and Guillemaud 2010; Cristescu 2015; Estoup et al. 2016) could help flesh out *C. perspectalis* invasion scenarios and clarify how the species spread across Europe and Asia Minor in less than ten years.

Compliance with Ethical Standards:

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Specimens sampled did not involve endangered nor protected species.

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Legends

Table 1. Genetic diversity statistics of native and invasive populations of *Cydalima perspectalis* based on the current study and Matošević et al. (2017).

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N°	Country	Populations	N	HTA1	HTA2	HTA3	HTA4	HTA5	HTB1	HTB2	HTB3	HTB4	HTB5	HTB6	HTC1	H	*h* (±SD)	**k**	*n* (±SD)
Native Range			132	40	3	1	2	1	43	29	6	1	1	1	4	12	0.755 (0.018)	4.25	0.0028 (0.0001)
N1	China	Huaibei	5						4	1						2	0.400 (0.237)	0.40	0.0003 (0.0002)
N2		Beijing	7	5					1	1						3	0.524 (0.209)	3.62	0.0024 (0.0008)
N3		Fuzhou	1	1												1	-	-	-
N4		Youxi	5	1					3	1						3	0.700 (0.218)	3.20	0.0021 (0.0011)
N5		Guiyang	8	1					4	3						3	0.679 (0.122)	2.29	0.0015 (0.0008)
N6		Xinyang	1						1							1	-	-	-
N7		Nanjing	5	3					1	1						3	0.700 (0.218)	4.60	0.0031 (0.0009)
N8		Shenyang	7	2					4	1						3	0.667 (0.160)	3.62	0.0024 (0.0008)
N9		Ordos	5	2	2									1		3	0.800 (0.164)	3.80	0.0025 (0.0013)
N10		Jinan	5	2		1			2							3	0.800 (0.164)	4.60	0.0031 (0.0008)
N11		Tai'an	8	2	1				3	2						4	0.821 (0.101)	4.43	0.0030 (0.0006)
N12		Dongying	3	1			1		1							3	1 (0.272)	5.33	0.0036 (0.0015)
N13		Wendeng	2							2						1	0	-	0
N14		Shanghai	14	5			1	1		7						4	0.659 (0.090)	4.59	0.0031 (0.0003)
N15		Kunming	8						4	2		1	1			4	0.750 (0.139)	0.93	0.0006 (0.0002)
N16		Lijiang	9						4	5						2	0.556 (0.090)	0.56	0.0004 (0.0001)
N17		Fuyang	12	4					3	1					4	4	0.773 (0.069)	5.98	0.0040 (0.0003)
N18		Hangzhou	8	6					1	1						3	0.464 (0.200)	3.25	0.0022 (0.0009)
N19		Lishui	6	5						1						2	0.333 (0.215)	2.67	0.0018 (0.0012)
N20	South Korea	Seoul	13						7		6					2	0.538 (0.060)	1.08	0.0007 (0.0001)
Invaded range			305	120	56		3		116	10						5	0.668 (0.012)	3.79	0.0027 (0.0001)
I1	Austria	Vienna	6						6							1	0	-	0
I2		Rankweil	2						2							1	0	-	0
I3	Belgium	Ghent	5	5												1	0	-	0
I4		Mechlin	1						1							1	-	-	-
I5		Vremde	3	1					2							2	0.667 (0.314)	4.67	0.0031 (0.0015)
I6	Bulgaria	Plovdiv	4	1	3											2	0.500 (0.265)	0.50	0.0003 (0.0002)
I7		Sofia	5	1					4							2	0.400 (0.237)	2.80	0.0019 (0.0011)
I8	Croatia	Vinica	6		1				5							2	0.333 (0.215)	2.67	0.0018 (0.0012)
I9		Osor	1						1							1	-	-	-
I10		Artatore	1	1												1	-	-	-
I11		Zagreb	5	3					2							2	0.600 (0.175)	4.20	0.0028 (0.0008)
I12		Višnjevac	5						5							1	0	-	0

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I13	Czech Rep.	Brno	5	2	2				1							3	0.800 (0.164)	3.40	0.0023 (0.0011)
I14	France	Grenoble	3	3												1	0	-	0

N°	Country	Populations	N	HTA1	HTA2	HTA3	HTA4	HTA5	HTB1	HTB2	HTB3	HTB4	HTB5	HTB6	HTC1	H	*h* (±SD)	**k**	*n* (±SD)
I15		Orléans	5	2					3							2	0.600 (0.175)	4.20	0.0028 (0.0008)
I16		Tours	7	4	1				2							3	0.667 (0.160)	3.62	0.0024 (0.0008)
I17		Bastia	4	2	2											2	0.667 (0.204)	0.67	0.0005 (0.0001)
I18		Saint Louis	9	6			1			2						3	0.556 (0.165)	3.33	0.0022 (0.0009)
I19		Strasbourg	4							4						1	0	-	0
I20		Paris	5	5												1	0	-	0
I21		Bordeaux	4		2				2							2	0.667 (0.204)	5.33	0.0036 (0.0011)
I22		La Rochelle	5	1	4											2	0.400 (0.237)	0.40	0.0003 (0.0002)
I23		Marcillac Vallon	2		1				1							2	1 (0.500)	8.00	0.0054 (0.0027)
I24		Roquefort-sur-Garonne	4	2	1				1							3	0.833 (0.222)	4.00	0.0027 (0.0012)
I25		Nantes	5	3	1				1							3	0.700 (0.218)	3.20	0.0021 (0.0011)
I26		Lagnes	11	7	2				1	1						4	0.600 (0.154)	2.80	0.0019 (0.0008)
I27	Georgia	Bzyb Valley	5	3	1				1							3	0.700 (0.218)	3.20	0.0021 (0.0011)
I28		Mtirala National Park	5	2	3											2	0.600 (0.175)	0.60	0.0004 (0.0001)
I29		Zugdidi	4		3				1							2	0.500 (0.265)	4.00	0.0027 (0.0014)
I30		Tbilisi	5		2				3							2	0.600 (0.175)	4.80	0.0032 (0.0009)
I31	Germany	Lorsch	5	3					2							2	0.600 (0.175)	4.20	0.0028 (0.0008)
I32		Kandern	4	4												1	0	-	0
I33		Kehl	3							3						1	0	-	0
I34		Weil Am Rhein	4	2			2									2	0.667 (0.204)	0.67	0.0005 (0.0001)
I35	Greece	Filiria	2						2							1	0	-	0
I36		Katerini	7						7							1	0	-	0
I37	Hungary	Harkány	5						5							1	0	-	0
I38		Hódmezővásárhely	5	3					2							2	0.600 (0.175)	4.20	0.0028 (0.0008)
I39		Budaörs	5	3					2							2	0.600 (0.175)	4.20	0.0028 (0.0008)
I40		Kőszeg	5	1					4							2	0.400 (0.237)	2.80	0.0019 (0.0011)
I41	Iran	Si Sangan National Park	3	1					2							2	0.667 (0.314)	4.67	0.0031 (0.0015)
I42	Italy	Bologna	3		3											1	0	-	0
I43		Ruta	3	1	1				1							3	1 (0.272)	5.33	0.0036 (0.0015)
I44		Legnaro	5						5							1	0	-	0
I45		Tregnago	4						4							1	0	-	0
I46		Cesa	2	1	1											2	1 (0.500)	1.00	0.0007 (0.0003)

I47		Florence	4		3				1							2	0.500 (0.265)	4.00	0.0027 (0.0014)
I48		Lucca	4	1	2				1							3	0.833 (0.222)	4.17	0.0028 (0.0013)
I49		Turin	4	2	2											2	0.667 (0.204)	0.67	0.0005 (0.0001)
I50		Viterbo	4	2	1				1							3	0.833 (0.222)	4.00	0.0027 (0.0012)

N°	Country	Populations	N	HTA1	HTA2	HTA3	HTA4	HTA5	HTB1	HTB2	HTB3	HTB4	HTB5	HTB6	HTC1	H	*h* (±SD)	**k**	*n* (±SD)
I51	Luxembourg	Luxembourg	2	1					1							2	1 (0.5)	7.00	0.0047 (0.0023)
I52	Netherlands	Giessen	9	1					8							2	0.222 (0.166)	1.56	0.0010 (0.0008)
I53		Boskoop	3	3												1	0	-	0
I54		Rotterdam	5	3					2							2	0.600 (0.175)	4.20	0.0028 (0.0008)
I55		Rhederbrug	3						3							1	0	-	0
I56	Portugal	Vila Nova de Cerveira	5	2	3											2	0.600 (0.175)	0.60	0.0004 (0.0001)
I57	Romania	Timişoara	3		2				1							2	0.667 (0.314)	5.33	0.0036 (0.0017)
I58	Russia	Solokhaul	4	3	1											2	0.500 (0.265)	0.50	0.0003 (0.0002)
I59		Komsomolsk	2	2												1	0	-	0
I60		Krasnodar	5	4	1											2	0.400 (0.237)	0.40	0.0003 (0.0002)
I61	Serbia	Belgrade	5	2					3							2	0.400 (0.237)	4.20	0.0028 (0.0008)
I62	Slovakia	Zvolen	5		5											1	0	-	0
I63		Bratislava	4						4							1	0	-	0
I64	Slovenia	Dobrovnic	2						2							1	0	-	0
I65		Nova Gorica	6	2					4							2	0.533 (0.172)	3.73	0.0025 (0.0008)
I66		Sečovlje	2						2							1	0	-	0
I67	Spain	Besalú	4	3	1											2	0.500 (0.265)	0.50	0.0003 (0.0002)
I68	Switzerland	Delémont	6	6												1	0	-	0
I69		Liestal	3	3												1	0	-	0
I70		Monteggio	3	3												1	0	-	0
I71	Turkey	Istanbul	5	3					2							2	0.600 (0.175)	4.20	0.0028 (0.0008)
I72		Yalova	2	1	1											2	1 (0.5)	1.00	0.0007 (0.0003)

The information provided for each location is population number (N°); number of individuals (N); haplotype number (H); haplotype diversity (*h*, with standard deviation SD); average number of nucleotide differences (k); and nucleotide diversity (*n*, with standard deviation SD).

Table 2. Results of the hierarchical AMOVA of COI-COII sequence data obtained from *Cydalima perspectalis* in its (a) native range and (b) invaded range.

Source of variation	df	Sum of squares	% of variation	Fixation index
(a) Native range				
Among groups	4	6.067	9.37	F_{CT} = 0.093*
Among populations within groups	15	7.725	8.43	F_{SC} = 0.094*
Within populations	112	35.693	82.20	F_{ST} = 0.178***
(b) Invaded range				
Among groups	3	11.091	11.61	F_{CT} = 0.116**
Among populations within groups	20	15.291	12.30	F_{SC} = 0.139***
Within populations	281	75.132	76.09	F_{ST} = 0.239***

Statistical probabilities were derived from 50,175 permutations; **p* value < 0.05, ***p* value < 0.01, ****p* value < 0.001. (a) In the native range, five groups were defined: (1) N8, N20; (2) N2, N9; (3) N3, N4, N6, N10, N11, N12, N13, N14; (4) N1, N7, N17, N18, N19; and (5) N5, N15, N16 (see Table 1 for population numbers). (b) In the invaded range, four groups were defined: (1) Germany, Netherlands, France, Italy, Belgium; (2) Switzerland, Spain, Portugal, Luxembourg; (3) Austria, Hungary, Czech Republic, Slovenia, Slovakia, Croatia, Greece, Serbia; and (4) Romania, Turkey, Russia, Bulgaria, Georgia, Iran.

Figure 1. Spread of *Cydalima perspectalis* across Europe and Asia Minor between 2007 (the year the species was first observed) and 2016. The two yellow stars indicate the first places that *C. perspectalis* was detected—in Germany (DEU) in 2007 (Krüger 2008). The gray dots represent the first observation(s) of the moth in each country, which were determined based on a literature review. Country abbreviations are as follows (listed by year of first moth observation): CHE: Switzerland (Leuthardt et al. 2010); NLD: Netherlands (Van der Straten and Muus 2010); FRA: France (Feldtrauer et al. 2009); GBR: United Kingdom (Salisbury et al. 2012); AUT: Austria; LIE: Liechtenstein; DNK: Denmark; ITA: Italy (Bella 2013); BEL: Belgium (Casteels et al. 2011); ROU: Romania (Gutue et al. 2014); TUR: Turkey (Hizal et al. 2012); HUN: Hungary (Sáfián and Horváth 2011); CZE: Czech Republic (Bella 2013); SVN: Slovenia (Seljak 2012); HRV: Croatia (Koren and Crne 2012); POL: Poland (Blaik et al. 2016); RUS: Russia (Gninenko et al. 2014); SVK: Slovakia (Bella 2013); GRC: Greece (Strachinis et al. 2015); ESP: Spain (Pérez-Otero et al. 2015); BGR: Bulgaria (Beshkov et al. 2015); SRB: Serbia (Vajgand 2016); MNE: Montenegro (Hrnčić et al. 2017); BIH: Bosnia and Herzegovina (Ostojić et al. 2015); MKD: Macedonia (Načeski et al. 2018); GEO: Georgia and Abkazhia (Matsiakh et al. 2018); UKR: Ukraine (Nagy et al. 2017); LUX: Luxembourg (Ries et al. 2017); PRT: Portugal (Maria da Conceição de Lemos Viana Boavida pers. comm.); ARM: Armenia (Shiroma Sathyapala pers. comm.); IRN: Iran (Mitchell et al. 2018); and SWE: Sweden (Bengtsson 2017).

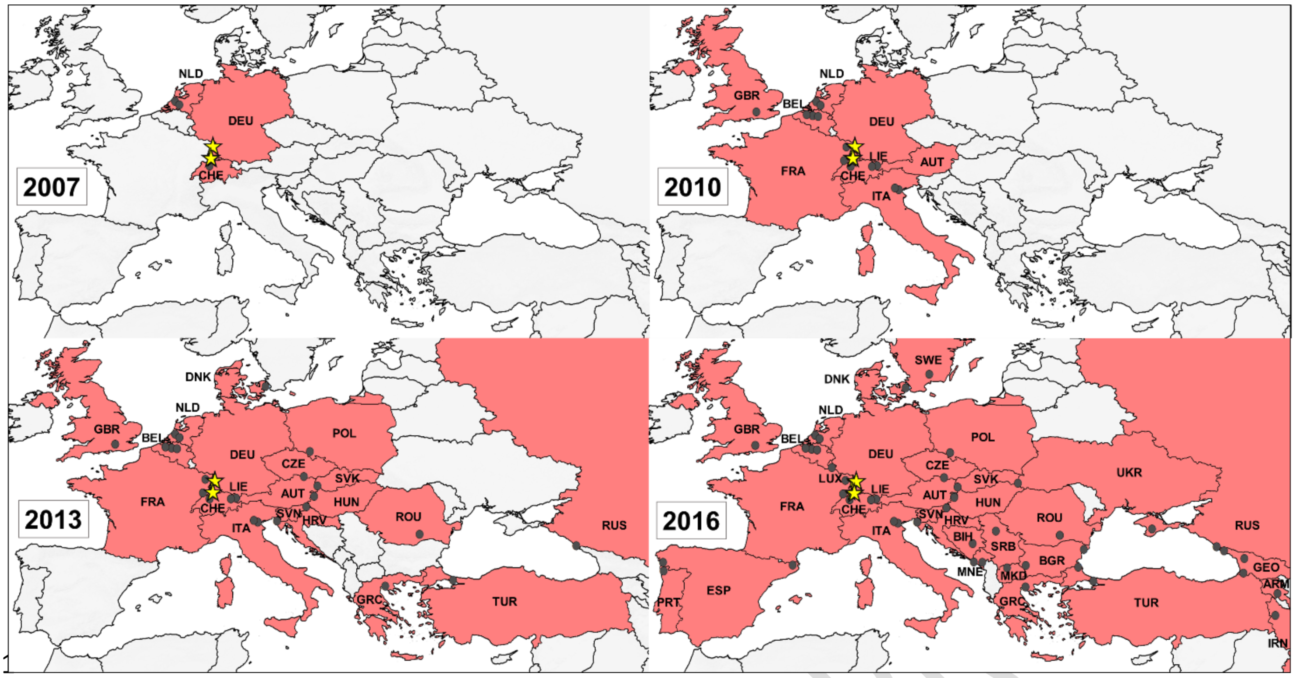


Figure 2a. Spatial distribution of *Cydalima perspectalis* COI-COII haplotypes in the moth's native range. The color codes indicate the color used in the haplotype network (see Figure 2b). The putative natural range of *C. perspectalis* was characterized based on records in the literature and moths collected as part of this study. The distribution of *Buxus* species was estimated based on Fang et al. (2011), and *Buxus* production in Chinese provinces was estimated using unpublished data provided by René Eschen.

2b. Network of *Cydalima perspectalis* COI-COII haplotypes based on the haplotype frequencies observed in the moth's native range. Each circle represents a haplotype (HTA1 to HTC1) and is labeled using a specific color. Circle size is proportional to the number of individuals. Each line between circles corresponds to a mutational step, and the small black circles are missing intermediate haplotypes.

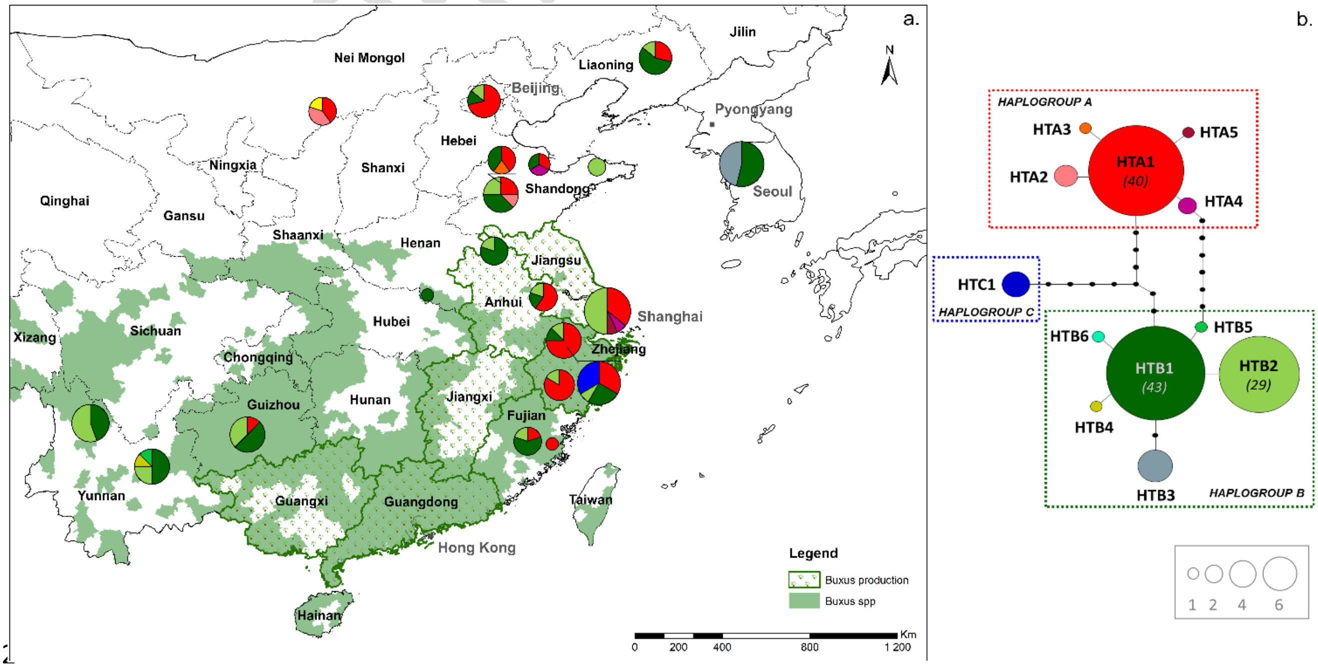
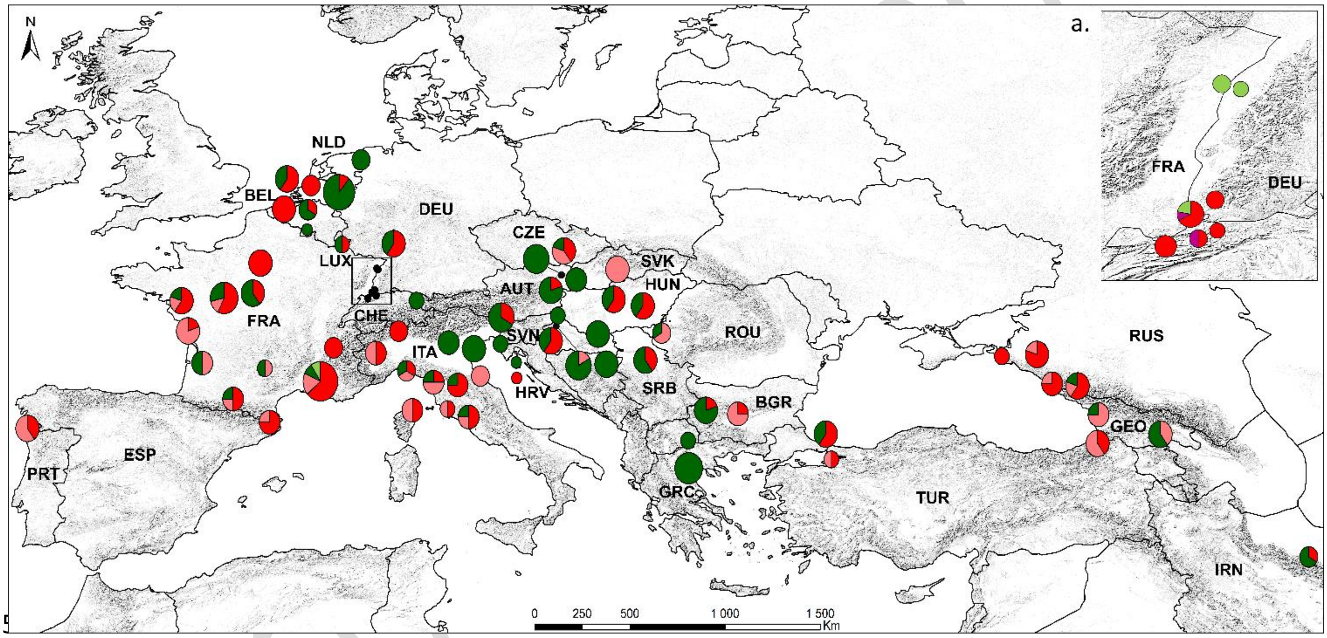
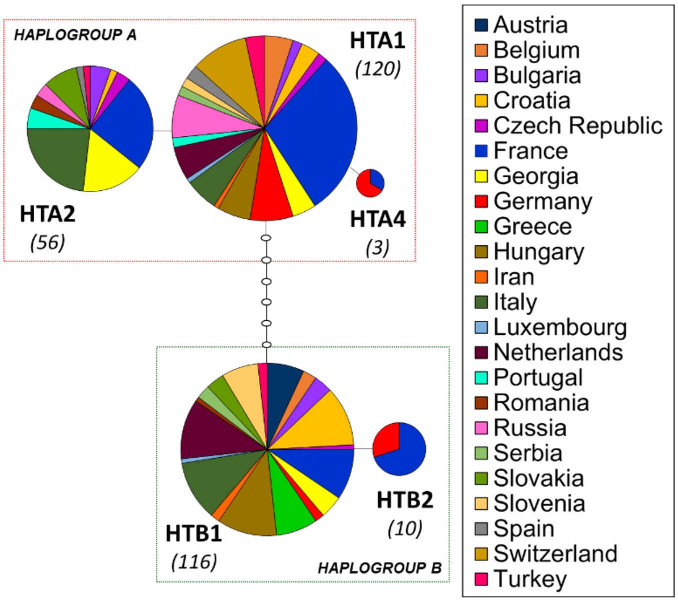


Figure 3a. Geographical distribution of *Cydalima perspectalis* COI-COII haplotypes in the moth's invaded range based on the results of the current study and Matošević et al. (2017). Circle size is proportional to the number of individuals. The color codes indicate the color used in the haplotype network (see Figure 2b). Country abbreviations are as follows: DEU: Germany; CHE: Switzerland; NLD: Netherlands; FRA: France; AUT: Austria; ITA: Italy; BEL: Belgium; ROU: Romania; TUR: Turkey; HUN: Hungary; CZE: Czech Republic; SVN: Slovenia; HRV: Croatia; RUS: Russia; SVK: Slovakia; GRC: Greece; ESP: Spain; BGR: Bulgaria; SRB: Serbia; GEO: Georgia; LUX: Luxembourg; PRT: Portugal; and IRN: Iran.

3b. Network of *Cydalima perspectalis* COI-COII haplotypes based on the haplotype frequencies observed in the moth's invaded range. Each circle represents a haplotype. Different colors represent sampled invaded countries by *Cydalima perspectalis*. Circle size is proportional to the number of individuals. Each line between circles corresponds to a mutational step, and the small empty circles are missing intermediate haplotypes.




HAPLOGROUP A
HTA1
(120)
HTA2
(56)
HTA4
(3)
HTB2
(10)
HTB1
(116)
HAPLOGROUP B
Austria
Belgium
Bulgaria
Croatia
Czech Republic
France
Georgia
Germany
Greece
Hungary
Iran
Italy
Luxembourg
Netherlands
Portugal
Romania
Russia
Serbia
Slovakia
Slovenia
Spain
Switzerland
Turkey

b.