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Mitochondrial and microsatellite DNA markers reveal a Balkan origin for the highly invasive horse-chestnut leaf miner *Cameraria ohridella* (Lepidoptera, Gracillariidae)

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Abstract

Biological invasions usually start with a small number of founder individuals. These founders are likely to represent a small fraction of the total genetic diversity found in the source population. Our study set out to trace genetically the geographical origin of the horse-chestnut leafminer, *Cameraria ohridella*, an invasive microlepidopteran whose area of origin is still unkown. Since its discovery in Macedonia 25 years ago, this insect has experienced an explosive westward range expansion, progressively colonizing all of Central and Western Europe. We used cytochrome oxidase I sequences (DNA barcode fragment) and a set of six polymorphic microsatellites to assess the genetic variability of *C. ohridella* populations, and to test the hypothesis that *C. ohridella* derives from the southern Balkans (Albania, Macedonia and Greece). Analysis of mtDNA of 486 individuals from 88 localities allowed us to identify 25 geographically structured haplotypes. In addition, 480 individuals from 16 populations from Europe and the southern Balkans were genotyped for 6 polymorphic microsatellite loci. High haplotype diversity and low measures of nucleotide diversities including a significantly negative Tajima's D indicate that *C. ohridella* has experienced rapid population expansion during its dispersal across Europe. Both mtDNA and microsatellites show a reduction in genetic diversity of *C. ohridella* populations sampled from artificial habitats (e.g. planted trees in public parks, gardens, along roads in urban or sub-urban areas) across Europe compared with *C. ohridella* sampled in natural stands of horse-chestnuts in the southern Balkans. These findings suggest that European populations of *C. ohridella* may indeed derive from the southern Balkans.

Keywords: *Aesculus hippocastanum*, Balkans, *Cameraria ohridella*, genetic bottleneck, invasion, phylogeography

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Introduction

More and more alien species are being introduced unintentionally outside their natural habitats because of the increasing rate of trade and travel in the world. Fortunately, only a small proportion of introduced aliens can invade their new environment, and establish themselves, developing dense populations. Among the different ecological, demographic and evolutionary factors that influence the outcome of a species introduction, the level of genetic diversity within alien populations has received increasingly attention as an important factor influencing the survival and

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adaptive potential of founders (Lee 2002; Facon *et al.* 2006).

Genetic variability among alien species varies depending on several factors. Populations of recently introduced aliens often show lower genetic diversity than do native populations (Puillandre *et al.* 2008). This reduced genetic diversity in nonnative ranges is likely to be the result of a 'founder effect' whereby the introduced individuals carry only a small fraction of genetic diversity of the source populations (Lockwood *et al.* 2007). If founder populations remain small over many generations they can lose most of their genetic variation via genetic drift, leading to high levels of inbreeding, a process known as a genetic bottleneck (Nei *et al.* 1975). On the other hand, populations of alien species may show a large fraction of the genetic variability of the native population when introductions involve many individuals (Roman 2006). Similarly, the genetic diversity sampled by an alien introduction will be high if founder populations come from different parts of a native range, which is already highly structured (Genton *et al.* 2005; Wilson *et al.* 2009).

Comparative analyses of the genetic variability of alien species between their native and introduced areas reveal a very diverse pattern (Cox 2004; Puillandre *et al.* 2008). Many alien insects show reduced genetic variability and evidence of genetic bottlenecks associated with the small number of founding individuals (Tsutsui *et al.* 2000; Schmid-Hempel *et al.* 2007; Puillandre *et al.* 2008). By contrast, many alien plants and aquatic invertebrates show high levels of genetic variability, (Holland 2001; Cox 2004), suggesting that initial populations are established by a large number of founding individuals and/or derived from introductions from different sources.

Determining the source locations of these alien invasive species is a key step in the development of invasive species management strategies. For example, it helps to identify potential biological control agents and pathways of introduction (Downie 2002; Gwiazdowski *et al.* 2006). However, the origin of many alien species remains uncertain because of the lack of historical data. For instance, of the 1514 species of terrestrial invertebrates considered as alien to Europe, 221 (i.e. 14.6%) are of unknown origin (Roques *et al.* 2008).

Among the alien invertebrate species of Europe, the case of the horse-chestnut leaf miner, *Cameraria ohridella* Deschka & Dimić (Lepidoptera: Gracillariidae) is of particular interest. This species was first discovered in Macedonia in 1984 (Deschka & Dimic 1986). Then in 1989, a second focal point was recorded in Austria, from where the moth is believed to have rapidly invaded most European countries, reaching as far west as Great Britain by 2000 (Pschorn-Walcher 1994; Gilbert *et al.* 2004, 2005).

The invasion of *C. ohridella* follows a stratified process of long-distance dispersal by human transport, combined with short distance diffusion by flight (Gilbert *et al.* 2005). Two nonexclusive hypotheses were proposed to account for the association of outbreaks with human populations: the higher risk of passive transportation between highly populated areas, and the higher urban densities of its main host tree, the horse-chestnut *Aesculus hippocastanum* L. (Gilbert *et al.* 2005). Indeed, *Ae. hippocastanum* is widely planted as an urban tree in most of Europe (its artificial dispersion started as early as 1576 from Vienna: Avtzis *et al.* 2007). By contrast, horse chestnut has a highly localized natural range in the southern Balkans, with sparse populations occurring in deep river gorges in central and northern Greece (with one eastern coastal population), also in Macedonia (former Yugoslavia) and Albania (Avtzis *et al.* 2007). *Aesculus hippocastanum* is actually considered as endangered in the IUCN plant red list of Albania (Vangjeli *et al.* 1997). In all regions where the moth is present, it maintains permanent outbreak densities, causing severe aesthetic damage to this highly valued ornamental and amenity tree (Freise & Heitland 2004). Concern has also been expressed for the survival of the relatively few horse chestnut native stands in the southern Balkans (37 were documented by Avtzis *et al.* 2007) because *C. ohridella* may seriously hamper natural regeneration (Thalmann 2003), although it does not appear to cause death of the trees.

Since the discovery of the moth, its area of origin has been a matter of heated debate. The first hypothesis, suggested by Deschka & Dimic (1986) and more recently supported by Grabenweger & Grill (2000) was that *C. ohridella* is probably a relict species, which survived the last glacial maximum period in the southern Balkans refugium. The main argument in favour of this hypothesis is the fact that *Ae. hippocastanum* itself is considered as a tertiary relict (Xiang *et al.* 1998). However, some of us (Pschorn-Walcher 1994; Holzschuh 1997; Kenis 1997; Kenis *et al.* 2005) also proposed four strong arguments against the Balkan origin of *C. ohridella*: (i) Considering the high dispersal capacities of the moth (Gilbert *et al.* 2005) and the high numbers of horse-chestnut trees planted for the last four centuries throughout Europe, why would it spread from the southern Balkans only now? (ii) Outbreaks in the Balkans have continued unabated for at least 25 years, and such continuous irruptions characterize invasive species rather than native species. Although most observations, even in the Balkans refer

to urban plantations recent surveys in natural stands of *Ae. hippocastanum* showed that populations of *C. ohridella* are unusually high for a leafminer (Grabenweger *et al.* 2005; Girardoz *et al.* 2007b). (iii) Until now, surveys in the Balkans have failed to identify specific natural enemies, in particular parasitoids, and parasitism rates are lower than those usually observed in native leaf miners (Grabenweger *et al.* 2005). (iv) The description of *C. ohridella* in 1986 represents a novel genus for Europe, suggesting an introduction. The remaining 70 *Cameraria* species occur in the eastern Palaearctic, North America and Asia (Davis 1983; De Prins & De Prins 2005).

Cameraria ohridella is also able to develop successfully on several maple species (*Acer* spp.) (Kenis *et al.* 2005) and is commonly found mining *Acer pseudoplatanus* L. around infested horse-chestnuts in Europe (Hellrigl 2001). This suggests that *C. ohridella* may have shifted from another host tree species (i.e. *Acer*) in the Balkans or elsewhere.

In a recent review, we discussed the origin of *C. ohridella* (Kenis *et al.* 2005) and considered an Asian origin as more likely, but we did not rule out the possibility that the moth could originate from the Balkans. We suggested performing a phylogeographic study on *C. ohridella* to assess the genetic heterogeneity of European populations. Indeed, DNA markers are a powerful tool to characterize the genetic variability of populations of alien species and assign them to potential sources in their native range (Gasparich *et al.* 1997; Tsutsui *et al.* 2001; Downie 2002; Scheffer & Grissell 2003; Hufbauer *et al.* 2004; Cognato *et al.* 2005; Grapputo *et al.* 2005; Eastwood *et al.* 2006; Gwiazdowski *et al.* 2006; Havill *et al.* 2006; Corin *et al.* 2007; Puillandre *et al.* 2008). Preliminary investigations using isozymes (Perny 1997) and RAPD-PCR (Kovács *et al.* 2000) identified very little variability among central European populations of *C. ohridella*, but these studies did not include populations from natural horse-chestnut stands.

In this paper, we use both mtDNA and newly generated microsatellites to test the hypothesis that *C. ohridella* originates from natural horse-chestnut stands in the southern Balkans and that only a small fraction of individuals were introduced into Western and Central Europe. If the hypothesis is correct, genetic diversity is expected to be significantly higher in the natural stands of *Ae. hippocastanum* in southern Balkans than in the rest of Europe, so we should find a significant loss of genetic diversity associated with the process of invasion.

Materials and methods

Sample collection

Samples of adults, larvae and pupae of *C. ohridella* (n = 486 individuals) were collected from 88 localities from 22 different European countries (Fig. 1, Appendix S1). A total of 239 individuals were sampled in Western and Central Europe. In addition, 247 individuals were collected in the native area of *Ae. hippocastanum* (southern Balkans: Albania, Macedonia and Greece) (Table 1).

Samples from mined leaves were handled as previously described (Lopez Vaamonde *et al.* 2003). Most samples were collected on *Ae. hippocastanum*, but 45 individuals were collected on *Ac. pseudoplatanus* and 12

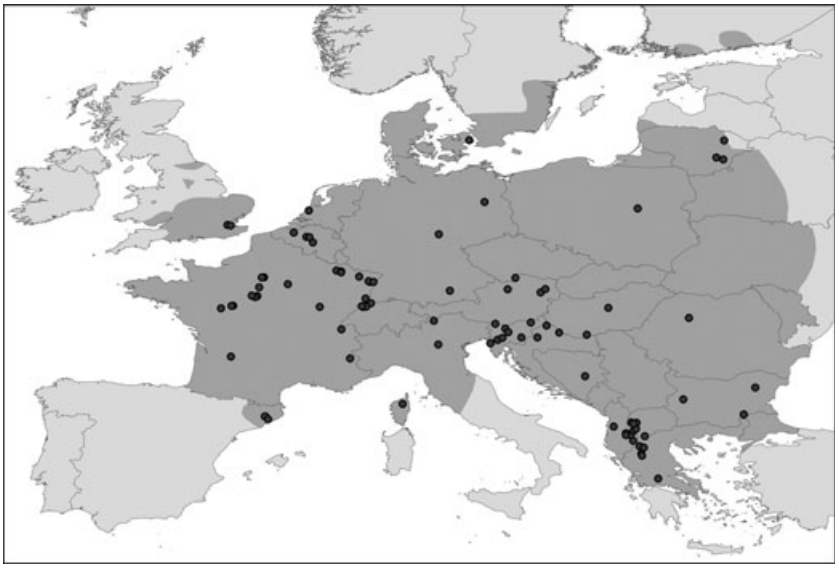

Fig. 1 Map showing the 88 localities sampled in our study (for details about localities see Appendix S1). In grey we show the known distribution range of *Cameraia ohridella*.

Table 1 Number of individuals, haplotype designation and genetic diversity for sampled populations grouped according to both geographical origin (Southern Balkans vs. Europe without Southern Balkans) and habitat status of collection sites (artificial habitats vs. natural stands) of *Cameraria ohridella*. The symbol ± indicates the standard deviation of each estimate

	No. individuals sampled	No. localities	No. haplotypes	Distribution of haplotypes	Haplotype diversity	Nucleotide diversity
Geographical region						
Southern Balkans	247	16	25	A(168) B(2) C(3) D(9) E(6) F(1) G(2) H(6) I(3) J(2) K(3) L(3) M(13) N(1) O(1) P(1) Q(1) R(5) T(2) U(1) V(1) W(3) X(6) Y(3) Z(1)	0.532 ± 0.039	0.00179 ± 0.00018
Rest of Europe	239	72	3	A(197) B(41) C(1)	0.292 ± 0.032	0.00091 ± 0.0001
Habitat Status						
Artificial habitats	351	79	8	A(295) B(42) C(1) D(4) E(6) I(1) J(1) F(1)	0.28 ± 0.029	0.00087 ± 0.0001
Natural stands	135	9	23	A(70) B(1) C(3) D(5) G(2) H(6) I(2) J(1) K(3) L(3) M(13) N(1) O(1) P(1) Q(1) R(5) T(2) U(1) V(1) W(3) X(6) Y(3) Z(1)	0.717 ± 0.041	0.00265 ± 0.00025

individuals on *Ac. platanoides* L (Appendix S1). We collected a single individual per leaf, per tree, and if possible, from 30 different trees per collecting site.

Mitochondrial DNA sequencing and analysis

DNA was extracted from larvae, adults (dry hind legs) or pupae using both the 'DNeasy tissue Kit' (Qiagen) and routine silica-based 96-well extraction automation protocol (Ivanova *et al.* 2006). Two hundred and ninety-nine individuals were sequenced with universal primer sequences HCO2198 and LCO1490 to yield a 633-bp fragment at the 5' end of the mitochondrial cytochrome *c* oxydase subunit I gene (COI) (Folmer *et al.* 1994) – a fragment also broadly used as a DNA barcode in various groups of animals (Hebert *et al.*, 2003). In addition, the same COI fragment was amplified for 187 other individuals using a slightly different primer set: LepF1/LepR1 (Hebert *et al.* 2004). The DNA extracts that did not amplify for the full-length gene fragment were selectively sampled and re-amplified with the two pairs of primers LepF1/MLepR1 and MLepF1/LepR1, targeting shorter DNA fragments and usually successfully amplifying specimens whose DNA was degraded with a high success rate (Hajibabaei *et al.* 2006). All PCR amplifications were performed according to the standard PCR reaction protocol used in CCDB (Hajibabaei *et al.* 2005). PCR products were checked on a 2% E-gel® 96 Agarose (Invitrogen). Unpurified PCR fragments obtained from the LepF1/LepR1 primer pair were sequenced in both directions; shorter fragments obtained with MLep primers were sequenced in one direction only. The sequencing reactions followed CCDB protocols (Hajibabaei *et al.* 2005), with products subsequently purified using Agencourt® CleanSEQ protocol (Agencourt).

Both strands of DNA were edited using BIOEDIT ver 7.0.5.3 (Hall 1999). No insertions, deletions or stop codons were present in the alignment. All sequences were truncated to the same length (633 bp) to eliminate missing data. Edited sequences of unique haplotypes were deposited in both the Barcode of Life Data Systems (BOLD) and GenBank (accession numbers: GQ143811 – GQ144316).

Records for all 486 barcoded specimens used in our analyses are gathered within the project 'Phylogeography of *Cameraria ohridella*' (code CAMER) in the Published Projects section of the BOLD; http://www.barcodinglife.org) (Ratnasingham & Hebert 2007). Information on specimen vouchers (field data and GPS coordinates) and sequences (nucleotide composition, trace files) are found in this project by following the 'view all records' link and clicking on the 'specimen page' or 'sequence page' links for each individual record.

Genetic differences among haplotypes were represented by a maximum parsimony network (Templeton *et al.* 1992) using TCS 1.21 (Clement *et al.* 2000). Sequence divergences were calculated using BOLD and Kimura's two parameter (K2P) as distance model.

Haplotype and nucleotide diversity (Nei 1987) (Table 1) and Tajima's D (Tajima 1989) were calculated using DnaSP version 4.10.9 (Rozas *et al.* 2003). We use ARLEQUIN (Excoffier *et al.* 1992) to test the concordance of our COI data with the predicted distribution under a model of sudden expansion (Rogers 1995).

Microsatellite protocols and analysis

Data were collected for each of six microsatellite loci for 480 individuals from 16 populations (nine artificial habitats and seven natural stands) from Europe (Appendix S2 and Table 2). Two hundred and eighty individuals from nine populations in artificial habitats and 200 individuals from seven populations in natural stands were genotyped (Appendix S2 and Table 2). The average number of individuals genotyped per population was 30 (21–39) (Appendix S2). Primer sequences and amplification protocols are given in Mari Mena *et al.* (2008).

Observed and expected heterozygosities and tests for departure from Hardy–Weinberg equilibrium (HWE) were calculated using ARLEQUIN 3.1 (Excoffier *et al.* 2005). Tests for linkage disequilibrium and allelic richness were calculated using FSTAT 2.9.3.2 (Goudet 2001). Micro-Checker (Van Oosterhout *et al.* 2004) was used to test for null alleles (Brookfield 1996) and identify possible scoring errors because of the large-allele dropout and stuttering.

For both mitochondrial and microsatellite data, the partition of genetic variability among populations and among groups of populations (southern Balkans vs. rest of Europe and natural stands vs. artificial habitats) was defined by analysis of molecular variance (AMOVA), (Excoffier *et al.* 1992) estimated by computing conventional F-statistics from haplotypes (for COI data) and allele frequencies (for microsatellite data), using Arlequin with 10 000 permutations. We further explored the distribution of genetic variation by estimating the number of populations represented by the sixteen sample locations genotyped using the software Structure (v.2.3; Pritchard *et al.* 2000). This approach uses a Bayesian, Monte Carlo Markov Chain (MCMC) approach to cluster individuals into groups while minimizing Hardy–Weinberg disequilibrium and gametic phase disequilibrium between loci within groups. The optimal number of populations (K) represented by the data can be calculated by comparing the estimated log probability of the data for different values of K (Pritchard *et al.* 2000). Initially, we performed a number of independent runs with different K values, iterations and burn-in periods to establish reliability of results. At the end we ran two independent runs with K values from 1 to 10, a burn-in period of 40 000 MCMC iterations and a data collection period of 1 million MCMC iterations. The independent runs produced consistent results for the same value of K.

Nonparametric statistics were used to test for differences in allelic richness measures between groups of populations (southern Balkans vs. rest of Europe and natural stands vs. artificial habitats), after checking for normality with SPSS Version 10 software (SPSS Inc.), as

Table 2 Summary of genetic variation (mean ± SD) of 6 microsatellite loci at 16 locations (9 artificial habitats and 7 natural stands) from Europe and the southern Balkans for *Cameraria ohridella* (see Appendix S2 for raw data)

	Artificial habitats							Natural stands						
	Berlin	Mézieres	Reading	Vienna	Tirana	Sofia	Tsotili	Ohrid	Kicevo	Karitsa	Radigoz	Stravaj	Monodendri	Ondria	Perivoli	Garska	Reka
N_A	5.33 ± 1.51	5 ± 1.41	7.5 ± 1.75	6 ± 2.53	4.33 ± 1.63	4.5 ± 1.76	4.5 ± 1.22	6 ± 2.61	4.33 ± 2.5	7.5 ± 2.43	7.83 ± 2.32	7.17 ± 4.02	7.83 ± 2.48	7.5 ± 1.52	8 ± 3.46	5.17 ± 1.47	
AR	5 ± 1.51	4.51 ± 1.3	4.94 ± 1.65	5.34 ± 1.96	4.17 ± 1.49	4.16 ± 1.43	4.19 ± 1.26	5.27 ± 2.03	4.09 ± 2.26	6.59 ± 2.06	6.99 ± 1.8	7 ± 3.87	7.2 ± 2.21	6.7 ± 1.32	7.15 ± 2.9	4.71 ± 1.35	
H_0	0.55 ± 0.3	0.59 ± 0.23	0.67 ± 0.33	0.72 ± 0.28	0.58 ± 0.13	0.42 ± 0.2	0.43 ± 0.18	0.57 ± 0.26	0.46 ± 0.27	0.59 ± 0.21	0.73 ± 0.16	0.56 ± 0.34	0.71 ± 0.18	0.7 ± 0.2	0.59 ± 0.35	0.3 ± 0.13	
H_E	0.6 ± 0.21	0.57 ± 0.16	0.6 ± 0.27	0.65 ± 0.16	0.58 ± 0.11	0.54 ± 0.1	0.56 ± 0.2	0.62 ± 0.24	0.52 ± 0.3	0.66 ± 0.24	0.75 ± 0.14	0.73 ± 0.19	0.75 ± 0.13	0.74 ± 0.09	0.71 ± 0.18	0.52 ± 0.2	

N_A, no. alleles; AR, allelic richness; H_0, observed heterozygosity; H_E, unbiased expected heterozygosity.

both mitochondrial and microsatellite datasets proved to have significantly nonnormal distributions.

Results

Mitochondrial DNA sequences

A total of 25 haplotypes were found among the 88 localities and 486 individuals analysed (Fig. 2, Table 1). Haplotype A was the commonest with 84.05% of individuals collected in artificial habitats and 51.85% of individuals collected in natural stands (Table 1). Haplotype B was the second commonest with 8.85% of all individuals collected. Out of a total 57 individuals collected on *Acer*, 13 (22.81%) had haplotype B. Eight haplotypes (F, N, O, P, Q, U, V and Z) were represented by a single individual and 68% of haplotypes are found exclusively in natural stands of *Ae. hippocastanum* in southern Balkans (Table 1, Fig. 2).

The parsimony-based network shows a star-like pattern, with one widespread haplotype (A) at the centre with derivatives connected to it by short branches (Fig. 3). Most of these derivative haplotypes are present only in natural stands of *Ae. hippocastanum* in the southern Balkans (Table 1, Figs 2–3).

Overall, genetic diversity of *C. ohridella* populations was higher in natural stands of horse-chestnuts than in artificial habitats (Table 1). The median number of haplotypes (range) was 1 (1–4) ($n = 79$ localities) for *C. ohridella* collected in artificial habitats and 2 (1–11) ($n = 9$ localities) for *C. ohridella* collected in natural stands. This difference was significant (Wilcoxon signed ranks test: $Z = -2.552$, $n = 87$, $P < 0.001$). Likewise, both haplotype and nucleotide diversity were higher in natural stands than in artificial habitats (Table 1). The highest genetic diversity was found in a natural stand in Perivoli (Greece), where we found 11 haplotypes for 32 *C. ohridella* individuals analysed (Appendix S1, Fig. 2). Haplotype diversity was significantly correlated to sampling effort (number of individuals sampled per population) (Spearman correlation: $r_S = 0.66$, $N = 87$, $P < 0.001$).

Tajima's D was negative ($D = -1.90064$) and deviated significantly from zero ($P < 0.05$) when all samples were combined. In addition, the model of sudden expansion (Rogers 1995) could not be rejected because of a high concordance of our COI data with the predicted distribution under a model of sudden-expansion (P/(Sim. Ssd ≥ Obs. Ssd)/ = 0.107 and (P(/Sim. Ssd ≥ Obs. Ssd)/ = 0.7342) for artificial habitats and natural stands respectively (5000 bootstraps).

Results of the AMOVA analyses are shown in Table 3. When populations were clustered in two groups, namely natural stands vs. artificial habitat samples, AMOVA showed that the greatest amount of total variation (68.73%) was accounted for by differences among individuals within populations. A much smaller but significant amount of variation (19.02%) was found among populations within groups, and finally variation among groups was found to be not significant. Likewise, when populations were clustered in southern Balkans vs. rest of Europe samples, variation among groups was found to be not significant.

Microsatellite variation

All six microsatellites were polymorphic within all populations with the exception of locus Ohrid2814, which was fixed for allele 103 in the population from Kicevo. The mean number of alleles detected per locus and per population varied from 4.33 in Tirana to 8 in Perivoli (Table 2). No linkage disequilibrium was observed for any pair of loci after Bonferroni correction. Therefore, further analyses were performed on multi-locus data from all six microsatellites. Significant deviation from HWE was observed at least in some of the six loci analysed in all sampling localities (Appendix S2). The presence of null alleles may have contributed to these departures from HWE. Significant evidence for null alleles was detected in several populations (Appendix S2).

Genetic diversity was significantly higher in natural stands than in artificial habitats, for mean number of alleles per locus per population ($P = 0.007$), allelic richness ($P = 0.004$) and expected heterozygosity ($P = 0.044$). Observed heterozygosity was higher in natural stands than in artificial habitats but the difference was not significant ($P = 0.244$) (Fig. 4). The outlier point is Garska Reka, a Macedonian natural stand, which shows relatively lower genetic diversity than other natural stands (see Discussion section). Similar results were obtained, when populations were clustered in southern Balkans vs. rest of Europe samples (data not shown).

Natural stand populations show a higher number of rare alleles than artificial habitat populations (Appendix S3). In addition, alleles present exclusively in a particular population (private alleles) were found at each locus. All natural stand populations and three artificial habitats (Tirana, Tsotili and Vienna) contained unique alleles. The mean number of private alleles (± SD) per locus per population was 2 (± 1) ($n = 7$ populations) for natural stands and 0.67 (± 1.12) ($n = 9$ populations) for artificial habitats.

Although the AMOVA revealed that most of the variation was found within sample locations, there was significant variation between both natural stands and artificial habitats (1.59%) and European and Southern Balkan populations (3.88%) (Table 3).

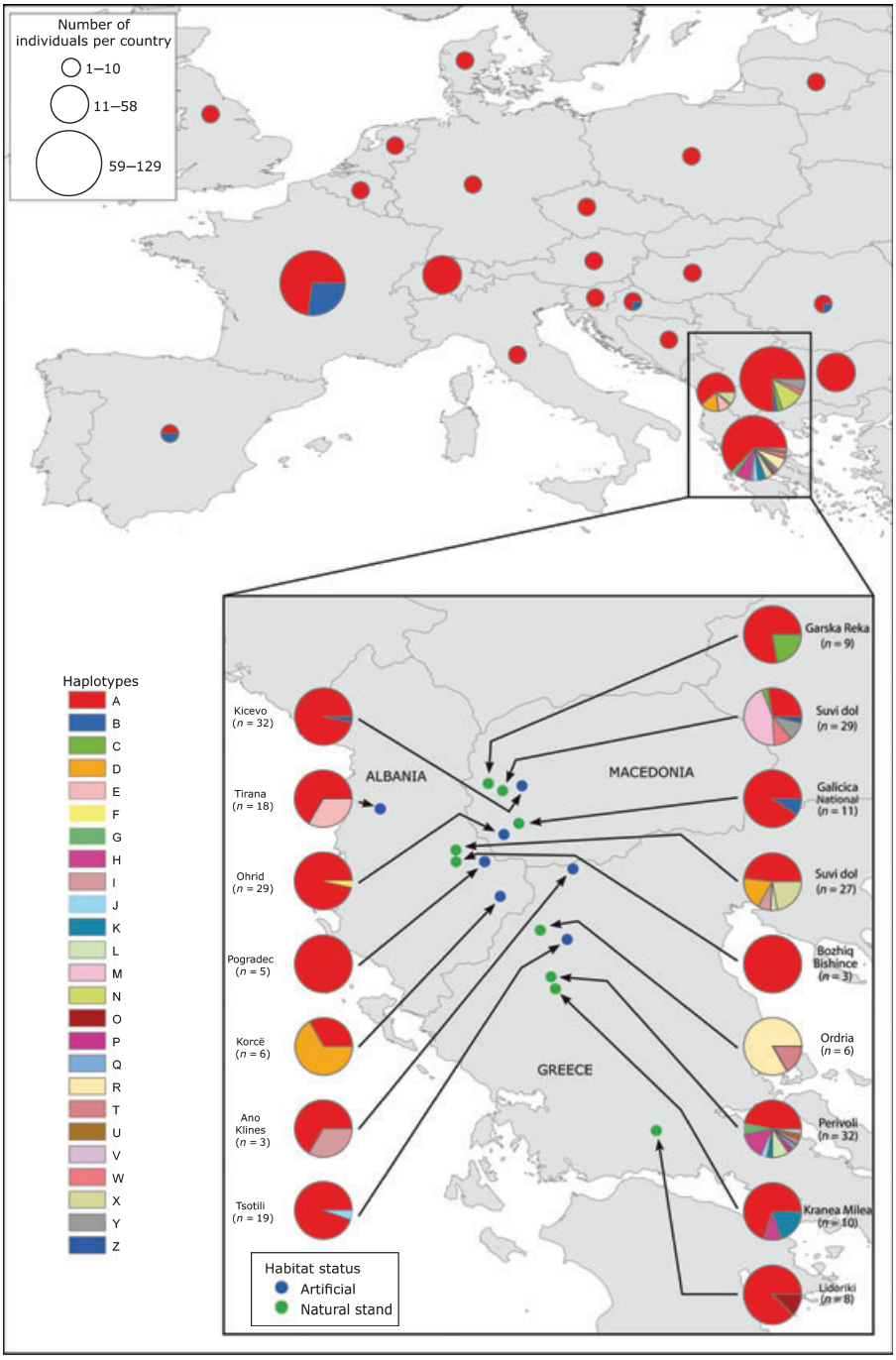

Fig. 2 Geographic distribution of the 25 haplotypes among the 22 sampled countries. Each pie chart represents a country and each haplotype is represented with a different colour. Number of individuals sampled per country is proportional to the size of each pie chart. For the southern Balkans, we illustrate the geographic distribution of the 25 haplotypes among 16 localities. Natural forests of *Aesculus hippocastanum* are represented by black circles and artificial habitats (i.e. planted trees in parks, gardens, along roads in urban or sub-urban areas) by blue circles. Number of individuals sampled per locality is indicated between parentheses.

Concerning the Structure analyses, the probability that the individuals from the 16 populations represent two groups was the highest (0.860), with substantial differences among populations with respect to assignment of individuals to these two groups (Table 4). One group contains all the natural populations plus two artificial habitats from the Balkans (Tirana and Tsotili), while the other groups the rest of the artificial habitat

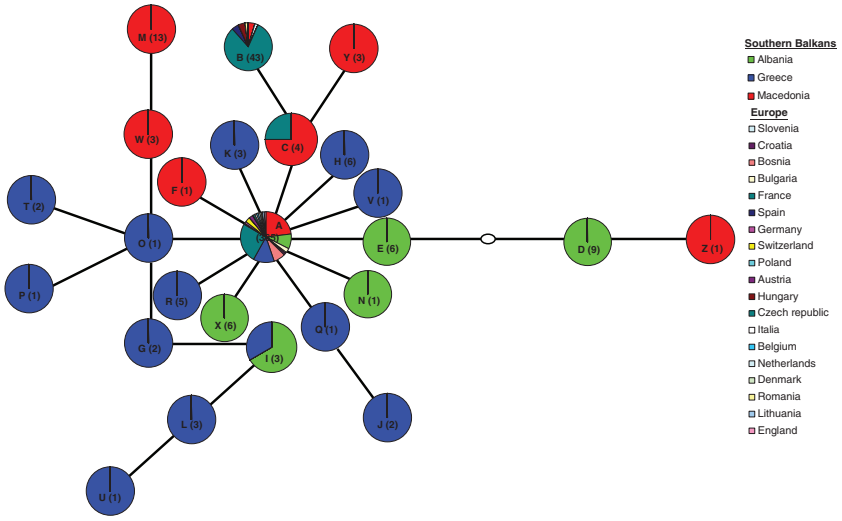

Fig. 3 Most parsimonious haplotype network of the 25 haplotypes, with corresponding letters. Colours indicate different countries where each haplotype is present. Haplotypes are connected with a 95% confidence limit. Each line in the network represents a single mutational change. Empty circles indicate intermediate, missing haplotypes. Number of individuals per haplotype is indicated between parentheses.

Table 3 Results of AMOVA test on mitochondrial and microsatellite markers

		mtDNA		Microsatellites	
	Source of variation	Variance components	% variation	Variance components	% variation
(a) Two groups (Natural stands/artificial habitats)	Among groups	0.02842	12.25^{NS}	0.03047	1.59*
	Among pops within groups	0.04411	19.02***	0.21625	11.27***
	Within populations	0.1594	68.73***	1.67176	87.14***
(b) Two groups (southern Balkans/rest of Europe)	Among groups	0.00962	4.37^{NS}	0.07536	3.88**
	Among pops within groups	0.05094	23.16***	0.19741	10.15***
	Within populations	0.1594	72.47***	1.67176	85.97***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; NS, not significant.

populations. The percentage of individuals assigned to each cluster is very high in all cases, except for the natural populations of Monodendri and Stravaj, whose individuals were assigned in almost equal proportions to both groups (Table 4).

Discussion

mtDNA diversity and founder effect

Most invasive insect species show a reduction in genetic diversity from native to invaded areas (Cox 2004; Puillandre *et al.* 2008). Our study shows a clear reduction in haplotype diversity from the southern Balkans to Western and Central Europe. This high genetic diversity found in the southern Balkans and the presence of a high number of unique haplotypes and private microsatellite alleles in natural stands of *Ae. hippocastanum* supports the hypothesis that the southern Balkans is likely to be the area of origin of *C. ohridella*. Indeed, the genetic diversity values observed for *C. ohridella* were greatest in the Balkan area and comparable to those obtained for other insects in their areas of origin. Thus, the mean percent divergence between haplotypes (0.487%) was within the range known for other Lepidoptera species (Wiemers & Fiedler 2007). In addition, the number of mitochondrial haplotypes (25 for 486 individuals) was moderate and total nucleotide diversity (0.141%) was relatively low when compared with other Lepidoptera species (Clarke & Whyte 2003; Vandewoestijne *et al.* 2004; Eastwood *et al.* 2006; Roe & Sperling 2007). Both moderate haplotype diversity and low nucleotide diversity are likely to be the result of a rapid demographic expansion (Hundertmark *et al.* 2002; Vandewoestijne *et al.* 2004). This idea is supported by both the significantly negative value of Tajima's D statistic and the concordance of our data with the distribution underlying the sudden expansion model of Rogers (1995). In addition, a clear indicative of a recent expansion of populations is the star-like pattern of the haplotype network. All this molecular evidence confirms that *C. ohridella* has undergone a well documented explosive

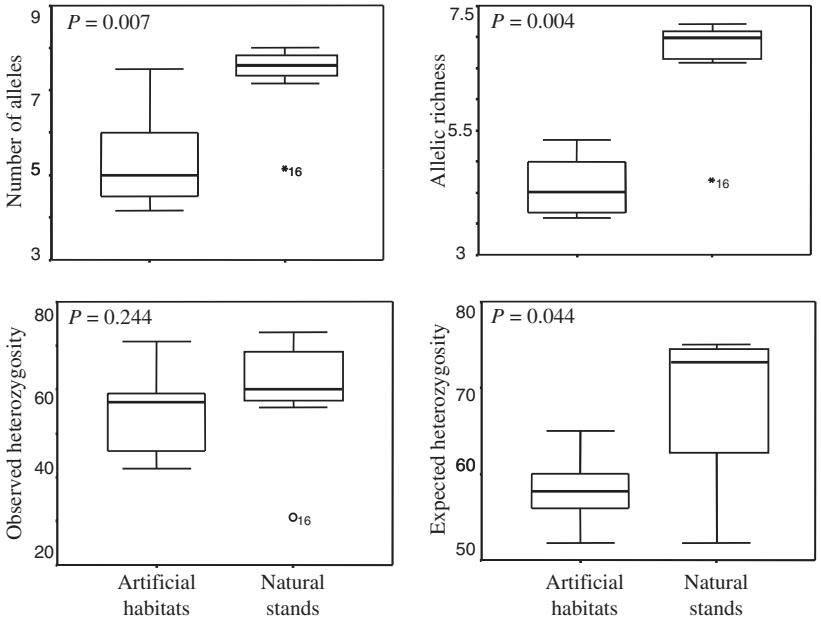

Fig. 4 Comparison of genetic diversity between *Cameraia ohridella* populations collected in artificial habitats and natural stands. *P*-values correspond to Kruskal–Wallis tests. The outlier (point number 16) is Garska Reka, a sample collected in a natural stand, which shows relatively low levels of genetic diversity compared with the rest of natural stands.

Table 4 Proportion of membership of individuals from each sample location in each of the two population clusters inferred from the Structure analysis

	Clusters	
	1	2
Artificial habitats		
Berlin	0.065	**0.935**
Mezieres	0.065	**0.935**
Reading	0.341	**0.659**
Vienna	0.199	**0.801**
Tirana	**0.881**	0.119
Sofia	0.283	**0.717**
Tsotili	**0.867**	0.133
Ohrid	0.213	**0.787**
Kicevo	0.165	**0.835**
Natural stands		
Garska Reka	**0.851**	0.149
Perivoli	**0.708**	0.292
Karitsa	**0.799**	0.201
Monodendri	**0.504**	0.496
Ondria	**0.667**	0.333
Radigoz	**0.633**	0.367
Stravaj	**0.549**	0.451

Proportions >0.5 are in bold.

population expansion starting from natural stands of *Ae. hippocastanum* in the southern Balkans and spreading to most Central and Western Europe (Gilbert *et al.* 2005). An alternative hypothesis would be that this high genetic diversity in the southern Balkans is simply the result of independent colonization events coming from outside southeastern Europe (i.e. an unknown source area in Asia and/or North America). However, most natural stands of *Ae. hippocastanum* are in remote mountainous areas, which are difficult to reach by terrestrial transport and therefore much more unlikely to be colonized several times independently, than are artificial habitats (i.e. public parks and gardens in urban areas). So, if the southern Balkans had been colonized by *C. ohridella* from abroad, we would expect to find higher genetic diversity in artificial habitats than in natural stands, but this is not the case.

Measures of genetic diversity are known to be sensitive to sample size. In this study we found that mitochondrial diversity was positively correlated to sampling effort. This is because of the fact that the mean number of individuals per locality sampled was 3.3 times lower for artificial habitats than for natural stands (15 individuals sampled per natural stand vs. 4.5 individuals sampled per artificial habitat). Therefore, it could be argued that the lower genetic diversity found in artificial habitats is just an artefact of low sampling effort. However, it is important to note that we sampled relatively high numbers of individuals for six artificial habitats: Mézières (30 individuals); Sofia (17); Kicevo (32); Ohrid (29); Tsotili (19); Tirana (18) (see Appendix S1). Despite the high numbers of individuals sequenced in those artificial habitats only 1–2 haplotypes were identified per locality. In addition, the total number of individuals sampled was relatively higher for artificial habitats (351) than natural stands (135). Likewise, the total number of localities sampled was much higher for artificial habitats (79) than natural stands (9). Furthermore, there is a low probability of sampling the same haplotype repeatedly for so many

localities even if sampling effort per locality is low. So we are confident about the reduced haplotype diversity found in artificial habitats relative to natural stands.

Microsatellite variation

The genetic diversity observed for this species in natural stands was similar to that of other Lepidoptera species in their native ranges (Endersby *et al.* 2006). As seen for haplotype data, populations of *C. ohridella* in Europe show some signs of a relatively mild population bottleneck: some private alleles have been lost, most likely via genetic drift, but without significant reduction in heterozygozity. Our microsatellite data show a significant reduction in the mean number of alleles per locus per population, allelic richness, and expected heterozygosity in artificial habitats, whereas values for observed heterozygosity were higher in natural stands than in artificial habitats though not significantly so. This could be explained owing to the fact that after a population bottleneck, allelic diversity is lost faster than heterozygosity, because of the loss of rare alleles, which has a low effect on heterozygosity (Cornuet & Luikart 1996; Rugman-Jones *et al.* 2007), especially when founder population is relatively large and the subsequent growth of the population is rapid (Nei *et al.* 1975). The deficits of heterozygotes to what is expected from Hardy–Weinberg equilibrium could also be caused by the presence of null alleles, strong inbreeding or by selection for or against a certain allele (Selkoe & Toonen 2006). This deficit of heterozygotes observed in our data is likely to be caused by the presence of nonamplifying alleles. The Macedonian natural stand Garska Reka showed unusually low levels of genetic diversity compared with other natural stands, which might be because of sampling related moths from only three different trees.

Sampling effort affects the number of alleles found. Our trend of higher frequency of rare (Appendix S3) and private alleles found in natural stands is not a sampling artefact since most populations had the same sample size.

In the Structure analysis, the fact that the greater proportions of individuals assigned to the 'artificial habitat' cluster are those from Vienna, Berlin, Mézières and Reading (66–94%) is likely to be because of a relatively recent introduction of a small population with common origin. Interestingly, two artificial habitats (Tirana and Tsotili) are grouped with the natural stands, which might indicate a recent introduction from nearby natural stands. On the other hand, the assignment of individuals to both of the inferred clusters in more even proportions (in particular Monodendri and Stravaj) could result from ongoing gene flow or from the greater time for population expansion. Taken together, the AMOVA and the clustering analysis support the hypothesis of a southern Balkan origin of *C. ohridella* in Europe. Further genotyping of more populations across Europe are needed to reconstruct with accuracy the colonization routes of *C. ohridella*.

Remaining questions about the invasion of C. ohridella

Although the high genetic diversity found in *C. ohridella* populations in natural stands of *Ae. hippocastanum* strongly suggests that the moth originates from these natural stands, some questions regarding its sudden expansion in Europe remain unanswered. Horse-chestnut has been planted as an ornamental tree in Balkan cities and villages for more than a century, sometimes less than 20 km from natural stands. Since *C. ohridella* invaded the whole Europe in only 20 years, why did it take so long to make the first jump from natural stands to planted trees? In all invaded regions, *C. ohridella* maintains outbreak densities and, thus, it is very unlikely that populations in urban areas in the southern Balkans would have remained unnoticed for a long time. The rapid spread of *C. ohridella* in Europe is usually considered to be associated with long distance dispersal events mediated by human activities (Gilbert *et al.* 2005). However, all natural horse-chestnut stands are, without exception, small, isolated areas in the southern Balkan mountains, from which *C. ohridella* was only likely to escape by natural dispersal. The fact that the moth took so long to reach planted trees in the vicinity of these stands would suggest that the natural dispersal capacity of the moth is very low (Augustin *et al.* 2009).

Cameraia ohridella is known to attack Norway maple and sycamore species in invaded areas, in particular when maple trees are in the vicinity of heavily attacked horse-chestnuts trees. The fact that a high percentage of individuals with haplotype B are found on *Acer* may suggest that there could be some degree of host-associated molecular divergence. Further sampling of moths on *Acer* will help to test this hypothesis.

The rather high population densities of *C. ohridella* in natural horse-chestnut stands and the apparent absence of specific natural enemies (Grabenweger *et al.* 2005; Kenis *et al.* 2005) are two characteristics usually more associated with alien than with native species. However, recent observations showed that population densities in natural stands in Albania and Macedonia are lower than on planted trees in the Balkans (R. Tomov and M. Kenis, unpubl. data). Furthermore, Girardoz *et al.* (2007c) suspect the occurrence of sibling species among the parasitoids of *C. ohridella*. Some of these

parasitoids may be more specific than previously thought and could be used as agents in a biological control programme. Studies are needed on the population dynamics and mortality factors in natural horse-chestnut stands where we found high genetic diversity in populations of *C. ohridella*. These data should be compared with similar investigations made in the invaded regions (Girardoz *et al.* 2007a,b), to better understand the extraordinary invasion success of the moth and, possibly, to develop new management strategies.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1 Localities, number of individuals, haplotype designation and genetic diversity for sampled populations of *Cameraria ohridella*.

Appendix S2 Summary of genetic variation of six microsatellite loci at 16 locations for *Cameraria ohridella*.

Appendix S3 The number of alleles per allele frequency category for all loci in samples from introduced (artificial habitats left column), and native (natural stands, right column) populations. The number of sampled individuals is given in parentheses.

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