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Are there autochthonous *Ferrissia* (Mollusca: Planorbidae) in the Palaearctic? Molecular evidence of a widespread North American invasion of the Old World

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Abstract

In the last few decades, findings of freshwater limpets belonging to the genus *Ferrissia* have been frequent and widely distributed throughout the Palaearctic and beyond. The widespread presence of a Nearctic alien taxon was proved, but no consensus was achieved about the possible existence of autochthonous *Ferrissia* taxa in the area, an occurrence which would be supported by the presence of gastropod fossils attributed to the genus throughout Eurasia and North Africa. In order to test the hypothesis of a possible persistence of autochthonous *Ferrissia* taxa in the Palaearctic to the present day, all the published data on the genetic diversity of *Ferrissia* populations occurring in the area were reviewed, expanding also the currently available dataset through dedicated sampling surveys in Italy, Malta and Spain. Here, based on currently published and novel large ribosomal subunit 16S rRNA (16S) and cytochrome oxidase sub-unit 1 (COI) sequences, the presence of the allochthonous *Ferrissia californica* in the whole Palaearctic was confirmed. Conversely, no evidence supporting the presence of autochthonous *Ferrissia* taxa was obtained. *Ferrissia californica* proved to be a highly invasive taxon (in spite of its extremely low genetic diversity) throughout the invaded regions, which is possibly related to the species' ability of asexual reproduction. Finally, the need to investigate the possible impact exerted by *F. californica* on the autochthonous Palaearctic biota is briefly stressed.

Keywords: *Biological invasions, cryptic invasions,* Ferrissia californica*, genetic paradox of invasions*

Introduction

Fossil records of the planorbid genus *Ferrissia* Walker, 1903 are known to occur throughout the Palaearctic region, with the genus being recorded from the Middle Miocene of Hungary, Austria, southern Germany and Switzerland (Schlickum 1976; Kókay 2006; Harzhauser et al. 2012, 2014b); from Pliocene deposits of France (Wautier 1974); and from the Early and Middle Pleistocene of Israel (Mienis & Ashkenazi 2011; Mienis & Rittner 2013). In addition, there is doubtful fossil evidence of the presence of *Ferrissia* sp. from interglacial Pleistocene and Holocene deposits in the Netherlands (Meijer 1987; Van Der Velde 1991) and Egypt (Bernasconi & Stanley 1994). Oddly, no evidence of the presence of extant *Ferrissia* limpets was collected in the Old World until the 20th century, when a few occurrences of representatives of this genus were found in France (Roger & Calas 1944), in Germany (Boettger 1949) and in Russia (reported by Son 2007). Afterwards, several findings occurred, and the reported taxa were identified mostly under the binomia *Ferrissia wautieri* (Mirolli, 1960) in Europe and *F. clessiniana* (Jickeli, 1882) in Africa and the Middle East. In fact, no consensus was reached on the taxonomic identity of Palaearctic *Ferrissia* until molecular evidence (e.g. Walther et al. 2006b,c; Marrone et al. 2011b) proved that all the studied *Ferrissia* populations occurring in the Palaearctic region belonged to a single allochthonous taxon of Nearctic origin, initially reported as *F. fragilis* (Tryon, 1863) and later as *F. californica* (Rowell, 1863), in accordance with the Principle of Priority of the International Code of Zoological Nomenclature (Christensen 2016).

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From the beginning of the 21st century, an ever-increasing body of evidence (Walther et al. 2006a, b and c, 2010; Beran & Horsak 2007; Son 2007; Semenchenko & Laenko 2008; Spyra 2008; Cilia 2009; Raposeiro et al. 2011; Marrone et al. 2011b, 2014; Tokinova 2012; Albrecht et al. 2013) has proved that the invasive North American *Ferrissia californica* widely invaded Europe, Asia and South America, while a molecular identification of the taxa occurring in Oceania (Greke 2011) and Africa (Brown 1965; Khalloufi & Boumaiza 2007) is, to date, lacking. However, based on the presence of Quaternary fossils of *Ferrissia* in the Palaearctic, the persistence of pre-Quaternary autochthonous *Ferrissia* populations in the region cannot be ruled out.

The present study aims to investigate the possible presence of autochthonous *Ferrissia* taxa currently living in the Palaearctic area, through the aggregate analysis of all the Palaearctic *Ferrissia* mtDNA sequences currently available in public repositories, and adding new data collected in the Central Mediterranean area.

Materials and methods

Ferrissia specimens (one to four from each sampled site) were collected during field surveys between 2008 and 2012 on the Italian mainland (including Lake Maggiore, the type locality of *Ferrissia wautieri*), Sardinia, Sicily, Malta and Spain (Table S1); the locations of the newly studied populations and of those for which published sequences are available were mapped using the QGIS software v. 2.18.2 (2016) (http://www.qgis.org) and are reported in Figure 1.

Water bodies were sampled with a 200-μm-mesh-sized hand net swept through the littoral vegetation and on the bottom of the studied water bodies. Collected samples were fixed *in situ* in 80% ethanol, and sorted under a stereomicroscope. The specimens, identified as *Ferrissia* sp. according to Girod et al. (1980), were then dipped in double-distilled water for 1 hour, and processed for DNA extraction using the Qiagen "DNeasy Animal Tissue Kit" following the manufacturer's instructions. Some specimens were deposited in the collection of the Museum of Natural Science of the University of Florence (Zoology Section "La Specola", Italy; see Table S1 for the catalogue numbers); others are stored in the molluscan collection of Federico Marrone at the University of Palermo (Italy) and are available for loan on request.

A partial sequence of the cytochrome oxidase subunit 1 (COI) gene was amplified using the universal primer pair LCO1490 and HCO2198 (Folmer et al. 1994). A portion of the large ribosomal subunit 16S rRNA gene was amplified using the primer pair "Skisto-1" and "Skisto-2" (see Marrone et al. 2011a). Composition of the polymerase chain reaction (PCR) mix and thermal cycles were performed according to Marrone et al. (2013).

After PCR amplification, 5 μL of each PCR product were separated by electrophoresis on a 2% agarose gel at 70 V for 1 h and visualised with a UV Transilluminator. When PCR products showed a clear and single band of the correct expected length, the whole PCR product was purified using the Exo-SAP purification kit and single-strand sequenced by Macrogen Inc. (Seoul, South Korea) with an ABI 3130xL (Applied Biosystems) sequencer with the
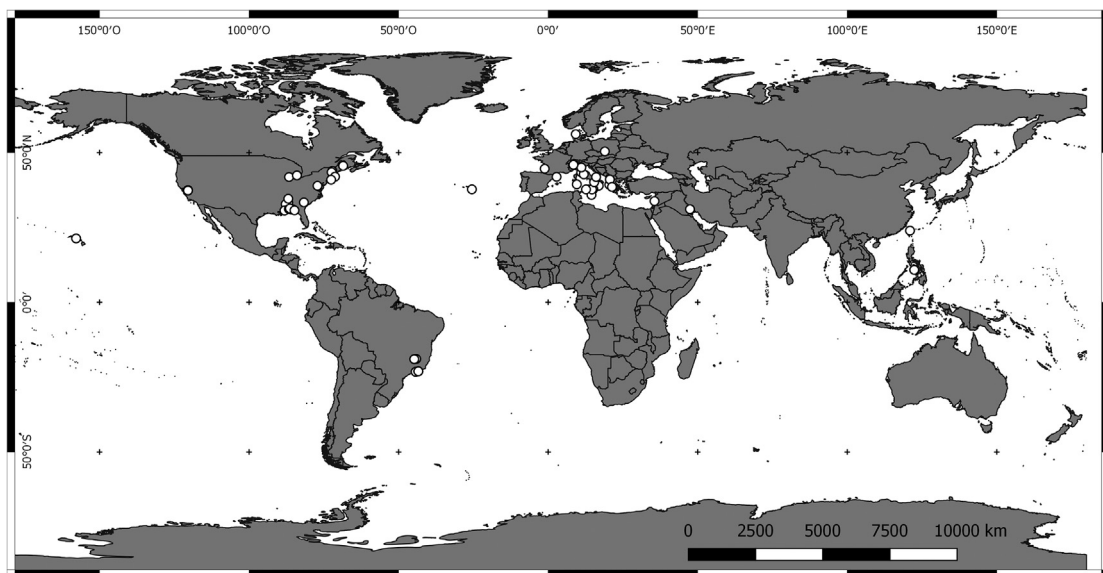

Figure 1. Map of the sampling sites of *Ferrissia* spp. for which mtDNA data are available.

same primers used for PCR amplification. Sequences were analysed and manually proofread with the DNA sequencing software Chromas v. 2.6.2 (Technelysium, Pty. Ltd. 1998, Queensland, Australia), and aligned with ClustalX v. 2.1 (Larkin et al. 2007).

In addition, in order to compare the new sequences obtained with those available for other countries, all of the 65 available COI and 17 16S rRNA *Ferrissia* spp. sequences from the Palaearctic and the Neotropical regions were downloaded from GenBank. Moreover, all the available *F. californica* (= *F. fragilis*) sequences from the Nearctic were downloaded for comparison, and eight *Ferrissia rivularis* (Say, 1917) and two *Pettancylus* sp. sequences were downloaded to be included as outgroups (see Albrecht et al. 2006; Walther et al. 2010; further information on the downloaded sequences is given in Table S1). The poor quality of the available COI sequences of *Ferrissia* from Denmark (Accession Number [AN] AY577502, erroneously labelled as *Acroloxus lacustris* (Linnaeus, 1758)) and COI and 16S rRNA sequences of *Ferrissia clessiniana* from Uganda (AN AY577509 and AY577469) did not allow us to include them in the analyses.

Analyses were conducted in two steps. Firstly, the molecular identification of the Palaearctic *Ferrissia* populations was performed through the reconstruction of phylogenetic trees based on all the available sequences for each mtDNA gene fragment, respectively. Afterwards, the COI genetic diversity observed in the Palaearctic and Neotropical biogeographical regions, where the single observed species (i.e. *Ferrissia californica*) proved to be allochthonous, were compared with that observed in the Nearctic, where *F. californica* finds its native range.

The genetic identification of the studied specimens and the reconstruction of the phylogenetic relationships among the taxa were performed with Bayesian inference (BI) and maximum likelihood (ML) methods as implemented in MrBayes v. 3.2.6 (Ronquist et al. 2012) and PhyMl v. 3 (Guindon & Gascuel 2003), respectively. As a measure of branch support, bootstrap values (Felsenstein 1985) were calculated with 1000 replicates in the ML trees. For both markers, two independent Markov Chain Monte Carlo analyses were run with 1 million generations (temp.: 0.2; default priors). Trees and parameter values were sampled every 100 generations resulting in 10,000 saved trees per analysis; in both analyses convergence was reached (Effective Sample Size (ESS) above 937.52 and 1161.86, respectively, for COI and 16S rRNA); 2500 trees were conservatively discarded as "burn-in". Nodes' statistical support of BI was evaluated by their posterior probabilities. The choice of the best evolutionary model was made using PartitionFinder v. 1.0.1 (Lanfear et al. 2012) according to the Akaike information criterion

(AIC; Akaike 1974). For both the COI and 16S rRNA datasets, BI and ML analyses were performed using a Hasegawa–Kishino–Yano (HKY) model of sequence evolution for molecular data.

A haplotype network for all the available COI sequences was constructed with the software Popart (v. 1.7, http://popart.otago.ac.nz), using the median-joining method (Bandelt et al. 1999); conversely, due to the low haplotype diversity observed for 16S rRNA, no network was built based on this marker.

Based on the output of the phylogenetic analyses, the sampled populations were partitioned into two groups: (i) autochthonous populations (USA; 36 COI and three 16S rRNA sequences); (ii) allochthonous populations (Asia, Europe and South America; 40 COI and 23 16S rRNA sequences).

Analysis of molecular variance (AMOVA) (Excoffier et al. 1992), mismatch analysis, haplotype diversity (Hd) and nucleotide diversity (π) were computed in order to detect possible population differentiation with DnaSP v. 5 (Librado & Rozas 2009). Furthermore, in order to estimate the significance of the pairwise differences in haplotype diversity between the two groups, the functions described by Thomas et al. (2002) and available at http://www.ucl.ac.uk/mace-lab/resources/software ("TEST_h_DIFF") were used through the statistical software "R" (v. 3.2.1, R Core Team 2015, available at https://www.R-project.org).

Results

Excluding the outgroups, 76 COI sequences (percentage of variable and parsimony-informative sites: 6.22% and 4.84%, respectively) and 26 16S rRNA sequences (percentage of variable and parsimony-informative sites, 0.63% and 0%, respectively) were analysed overall.

Both phylogenetic methods (ML and BI) showed the presence of a well-supported clade (1.00 posterior probability, pp, and 84% bootstrap support, bs, in the Bayesian and ML analyses for the COI marker, and 1.00 pp and 85% bs for the 16S rRNA marker; see Figures 2 and 3), which includes all the autochthonous North American *Ferrissia californica* populations along with all the *Ferrissia* sp. specimens to date investigated from the Palaearctic and Neotropical regions (Figures 2 and 3). Conversely, the North America *F. rivularis* clade is separated from the *F. californica* one by deep genetic distances and well-supported nodes, in accordance with its status of *bona species* (Walther et al. 2010).

Overall, 21 COI haplotypes were obtained (Table S1). The haplotype network (Figure 4) indicates the existence of three main and widespread haplotypes, and some rarer haplotypes shared by
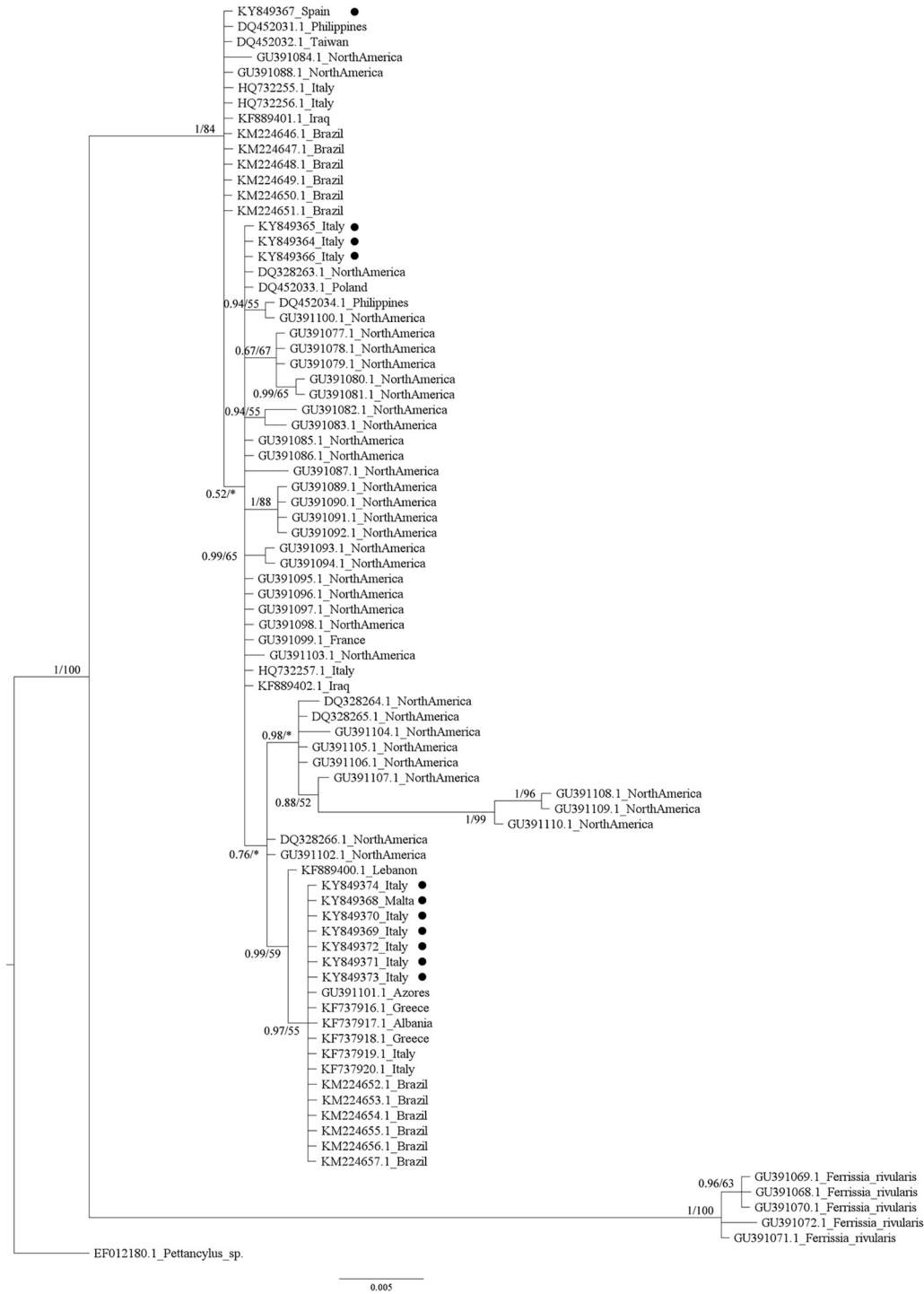
Figure 2. Bayesian phylogram (95% majority rule consensus tree) of the focal *Ferrissia* spp. using the mitochondrial cytochrome oxidase 1 (COI) dataset. *Ferrissia rivularis* and *Pettancylus* sp. were used as outgroups. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI) and bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. •: Novel sequences.

one or a few individuals mostly restricted to single continents. Most of the haplotypes belong to, or include at least one of, the North American samples excepting the haplotypes H11 and H12, which were observed solely in "South America and Europe" and in "Asia", respectively.

The comparison of the two groups showed that both nucleotide and haplotype diversity is higher in the
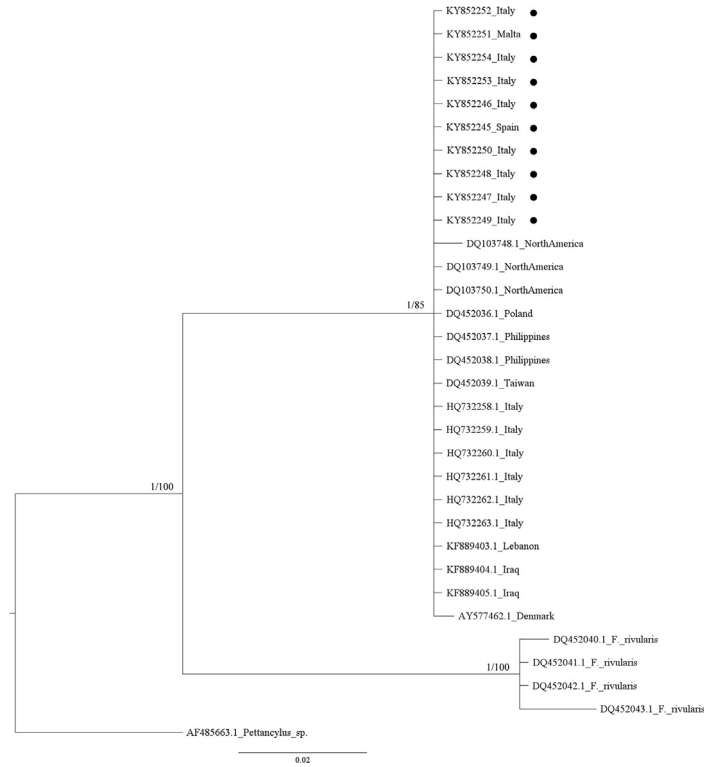
Figure 3. Bayesian phylogram (95% majority rule consensus tree) of the focal *Ferrissia* spp. using the mitochondrial 16S ribosomal subunit (16S rRNA) dataset. *Ferrissia rivularis* and *Pettancylus* sp. were used as outgroups. Node statistical support is reported as nodal posterior probabilities (Bayesian Inference of phylogeny, BI) and bootstrap values (Maximum Likelihood, ML). Asterisks indicate a bootstrap support value lower than 50. •: Novel sequences.
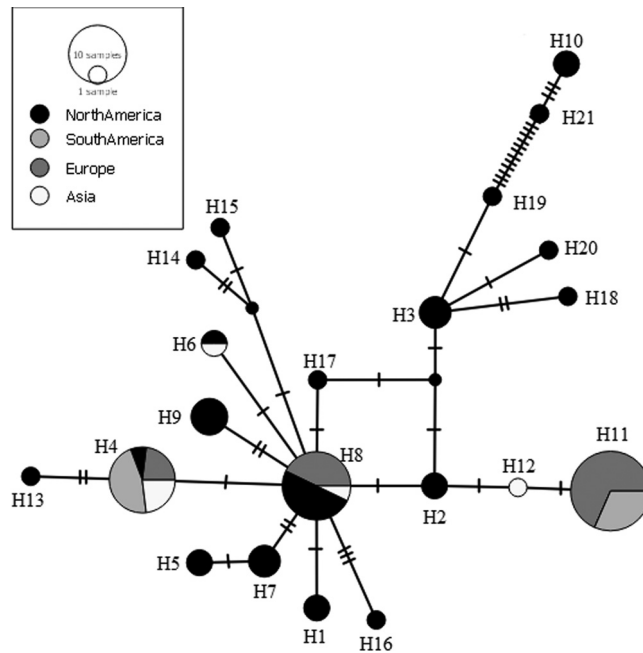

Figure 4. Median-joining haplotype network based on a fragment of mtDNA cytochrome oxidase sub-unit 1 (COI). Dashes indicate substitution steps.

"autochthonous" group than in the "allochthonous" one, and this applies for both markers (Table I). AMOVA test results (Table II) show that the percentage of variation among the two geographical groups is 17.37%, and within the groups it is 82.63%, with an Fixation index (F_{ST}) value of 0.173 and a p value of 0.000. The mismatch analysis showed an observed number of pair-wise differences which differs significantly from the expected values (Sum of Square Deviations (SSD) p value = 0.011; raggedness index = 0.028; raggedness p value = 0.039, Figure 5).

Table I. Nucleotide diversity (Pi) and haplotype diversity (Hd) of the "autochthonous" and "allochthonous" *Ferrissia* groups based on the mitochondrial cytochrome oxidase 1 (COI) and 16S ribosomal subunit (16S rRNA) gene fragments.

Group	(Pi)	(Hd)
COI		
Autochthonous	0.009	0.941
Allochthonous	0.003	0.669
16S rRNA		
Autochthonous	0.004	0.667
Allochthonous	0	0

Table II. AMOVA F_{ST} results based on 578-bp-long fragments of the mtDNA COI gene. "Va" is the variance among groups, "Vb" is the variance among populations within groups.

Source of Variation	df	Sum of squares	Variance components	Percentage of variation
Among groups	1	16.419	0.385 Va	17.370
Within groups	74	135.489	1.830 Vb	82.630
Total	75	151.908	2.216	

Fixation index (F_{ST}): 0.173; p value: 0.000.

Discussion

In spite of the fossil evidence indicating the presence of autochthonous *Ferrissia* species in the Palaearctic until the late Pleistocene or early Holocene, the tree topologies obtained using fragments of both COI and 16S rRNA mtDNA markers strongly support the molecular homogeneity of all the current Eurasian (and South American) *Ferrissia* populations, and their conspecificity with *F. californica* from North America.

The AMOVA results and the topology of the haplotype network show the presence of a substantially higher diversity within the group of autochthonous (i.e. Nearctic) populations than in the group of the allochthonous (i.e. Palaearctic and Neotropical) ones. Moreover, the haplotypes found in North America are also present in South America and Eurasia, which host an impoverished subset of the molecular diversity scored in the Nearctic. The only exceptions are the H11 and H12 haplotypes, occurring in Europe, Asia and South America, which are, to date, absent from the Nearctic area. However, the lack of these haplotypes in North America is likely to be ascribed to subsampling of *Ferrissia californica* in its native range (e.g. along the west coast of the USA, cf. Walther et al. 2010), and it is likely that the H11 and H12 haplotypes could be found there when a higher sampling effort is performed. This evidence supports the allochthnous status of *Ferrissia californica* in Eurasia, and its North American origin. Moreover, the mismatch analysis suggests that the entire population of *Ferrissia californica* is probably under recent demographic expansion.

The occurrence of *Ferrissia californica* out of its native range may be ascribed to the unwitting anthropochorus dispersal of this species along with the aquarium trade,
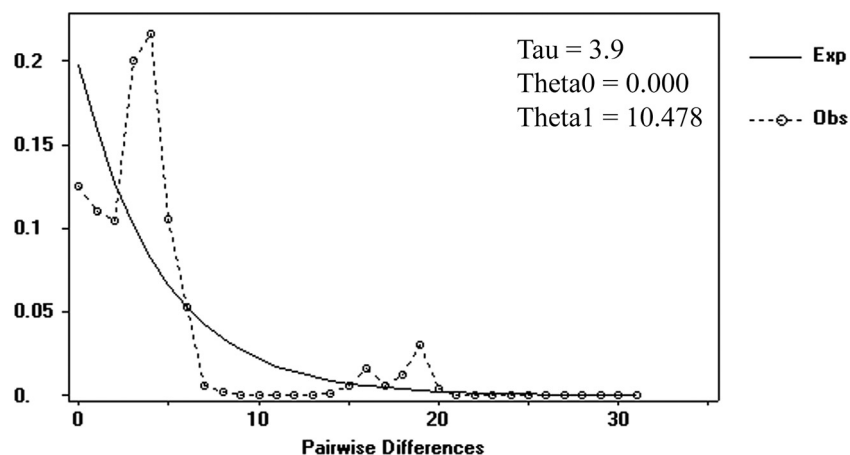

Figure 5. Result of the mismatch distribution analysis based on a fragment of mtDNA cytochrome oxidase sub-unit 1 (COI). The dotted line represents the observed frequency distribution of pairwise differences; the curve represents the expected ones.

followed by its release by the aquarists through aquarium waste disposal – e.g. Duggan (2010) reports the finding of *F. californica* (as *F. fragilis*) in the sediments of aquariums in New Zealand. It has been verified that the aquarium trade is responsible for the establishment of a large number of species into non-native areas (e.g. Mackie 1999; Rixon et al. 2005). In addition, there are several well-documented examples showing the global invasiveness of "aquarium-dispersed" gastropod species, such as *Potamopyrgus antipodarum* (Gray, 1843), *Melanoides tuberculata* (Müller, 1774) and *Haitia acuta* (Draparnaud, 1805) which invaded almost every freshwater ecosystem of the world in less than two centuries (e.g. Dillon et al. 2002; Taylor 2003; Bousset et al. 2004; Wethington & Lydeard 2007; Albrecht et al. 2009; Naser et al. 2011; Ladd & Rogowski 2012; Collado 2014; Marrone & Naselli-Flores 2015).

Despite the low genetic diversity observed in non-native areas, *Ferrissia californica* displays a noteworthy invasive capacity in markedly different areas with suitable habitats and climates, from the temporary ponds of the Mediterranean area through rivers and lakes of Northern Europe, to tropical environments in the Philippines and Brazil (e.g. Raposeiro et al. 2011; Tokinova 2012; Albrecht et al. 2013; Marrone et al. 2014; Lacerda et al. 2015; and references therein). This is in line with the so-called "genetic paradox of invasions" (Roman & Darling 2007; Caron et al. 2014; Schrieber & Lachmuth 2017), based on the disappointments of the expectation that a greater invasive capacity should be attributed to higher genetic diversity. In fact, rather than hinder invasion, bottlenecks that occur during man-mediated introduction events might increase the invasive capacity of the invader by depleting deleterious alleles so that even inbred individuals have a high fitness (see Estoup et al. 2016). Furthermore, those taxa which have a non-gonochoric reproduction mode (as in the case of *Ferrissia californica*) are often advantaged during the colonisation of new habitats because they have a higher potential for demographic growth (and subsequent monopolisation of resources) compared to those having a gonochoric reproduction (see Incagnone et al. 2015, and references therein). Once it invaded a new region, *Ferrissia californica* proved to be able to disperse locally using other physical and/or biological vectors. Genetic diversity and life-history traits (e.g. reproduction mode, see above) are known to determine the invasive potential of a species and its interactions with other taxa, and the combination of the two factors seems to have favoured the spreading of *Ferrissia californica* throughout invaded continents.

In conclusion, we could not find support for the current presence of indigenous *Ferrissia* species in the Old World, and it seems that the autochthonous taxa previously occurring in the area did not survive the Pleistocene or the early Holocene epochs. However, the possible presence of autochthonous *Ferrissia* species in North Africa and the Middle East, where Holocene subfossil records of *Ferrissia* were found (see also Marrone et al. 2014), cannot currently be ruled out. Thus, retrieving and analysing samples from these areas is now crucial in order to assess the possible presence of autochthonous *Ferrissia* in the Palaearctic, and to test the possible synonymy of *F. clessiniana* (which was described from northern Egypt) with *F. californica*.

Furthermore, taking into account the current lack of studies focusing on the impact exerted by *Ferrissia californica* on the autochthonous biota of invaded water bodies, future studies should ideally focus on this issue, evaluating whether, where, and how it might be necessary to monitor and manage this species in order to minimise the possibility of this documented "cryptic invasion" to evolve into an "evident trivialisation" of the Palaearctic biodiversity.

Supplemental data

Supplemental data for this article can be accessed here.

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