

Cryptic invasion in Southern Europe: The case of *Ferrissia fragilis* (Pulmonata: Ancyliidae) Mediterranean populations

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Abstract: Four populations of the North American ancyliid gastropod *Ferrissia fragilis* have been found in Southern Italy, thus expanding the known Eurasian distribution range of this invasive gastropod to the Mediterranean area. Both mitochondrial markers used for the molecular identification of the sampled specimens (16S and COI) showed a modest to absent haplotypic diversity in the studied *Ferrissia* populations from Europe and Asia, and their perfect identity with some of the haplotypes observed in North American *F. fragilis* populations. In the light of the scarce molecular diversity observed in the whole Palaearctic area and of the growing evidences for the ongoing spreading of the species in Europe, the occurrence of a single invasion event from North America possibly linked with aquarium plant trade followed by a rapid spreading of the species in Eurasia is suggested. The record of *F. fragilis* in Southern Italy caused some doubts on the presence of autochthonous *Ferrissia* populations in Europe and on the actual existence of *Ferrissia wautieri*. A molecular analysis of *Ferrissia* specimens from the *loci typici* of *F. wautieri* (Northern Italy) and *F. clessiniana* (Egypt) is definitely opportune.

Key words: Ancyliidae; *Ferrissia fragilis*; Italy; biological invasions; molecular identification; aquarium trade

Introduction

It is well known that aquatic habitats support relatively high numbers of alien species (Pyšek et al. 2010), and that these may be introduced more or less intentionally. The aquarium trade is one important, and often overlooked, possible "invasion path", and aquariumists may be effective carriers for allochthonous fauna and flora (Padilla & Williams 2004; Duggan 2010). In Southern Italy, in spite of the scarcity of studies dealing with the inland water systems, several examples of well-established allochthonous freshwater species that likely arrived through aquarium trade are available, e.g., the molluscs *Melanoides tuberculatus* (O.F. Müller, 1774) and *Haitia acuta* (Draparnaud, 1805) (Cianfanelli et al. 2007), the crayfish *Procambarus clarkii* (Girard, 1852) (D'Angelo & Lo Valvo 2003) and the African clawed toad *Xenopus laevis* (Daudin, 1802) (Faraone et al. 2008; Lillo et al. 2011), thus confirming the actual threat that a poorly regulated pet trade constitutes for local biocoenoses and biota.

The finding in Sicily and Calabria (Southern Italy) of some populations belonging to the ancyliid gastropod genus *Ferrissia* Walker, 1903 allowed us to test for the possible presence of an allochthonous *Ferrissia* species in Southern Europe. In fact, the genus *Ferrissia* comprises an uncertain number of species of primarily Holarctic distribution and a strong taxonomical uncertainty currently hampers a sound reconstruction of the actual diversity of the genus and the singling out of

the allochthonous *vs.* autochthonous populations (e.g., Albrecht et al. 2004, 2006; Walther et al. 2006a; Dillon & Herman 2009).

Representatives of the genus *Ferrissia* are known to occur throughout the European continent (Bank 2010). Walther et al. (2006b, c), based on molecular data, reported the presence of some populations of the North American invasive species *Ferrissia fragilis* (Tryon, 1863) in Denmark, Germany and Poland, while the taxonomical status of the other European populations is still under debate. Accordingly, the presence of a presumptive European autochthonous taxon, *Ferrissia wautieri* (Mirolli, 1960) is reported for some countries, the presence of the African/Asian *F. clessiniana* (Jickeli, 1882) for others, and the presence of the North American *F. fragilis* in the others. Furthermore, it is currently uncertain whether one or more *Ferrissia* species do actually occur in Europe.

Although some fossil records dating back to the Pliocene and the Pleistocene attributed to the genus *Ferrissia* testify for an ancient occurrence of the genus in Europe (van der Velde 1991 and references therein), its presence was completely overlooked till the twentieth century, when some specimens were collected and stored in the Zoological Museum of St. Petersburg (Russian Federation) and others were collected in France and Germany (Walther et al. 2006b; Son 2007). In the further decades, an increasing amount of records throughout the whole Europe peaked with the description of a new species, *F. wautieri*, which was considered to be

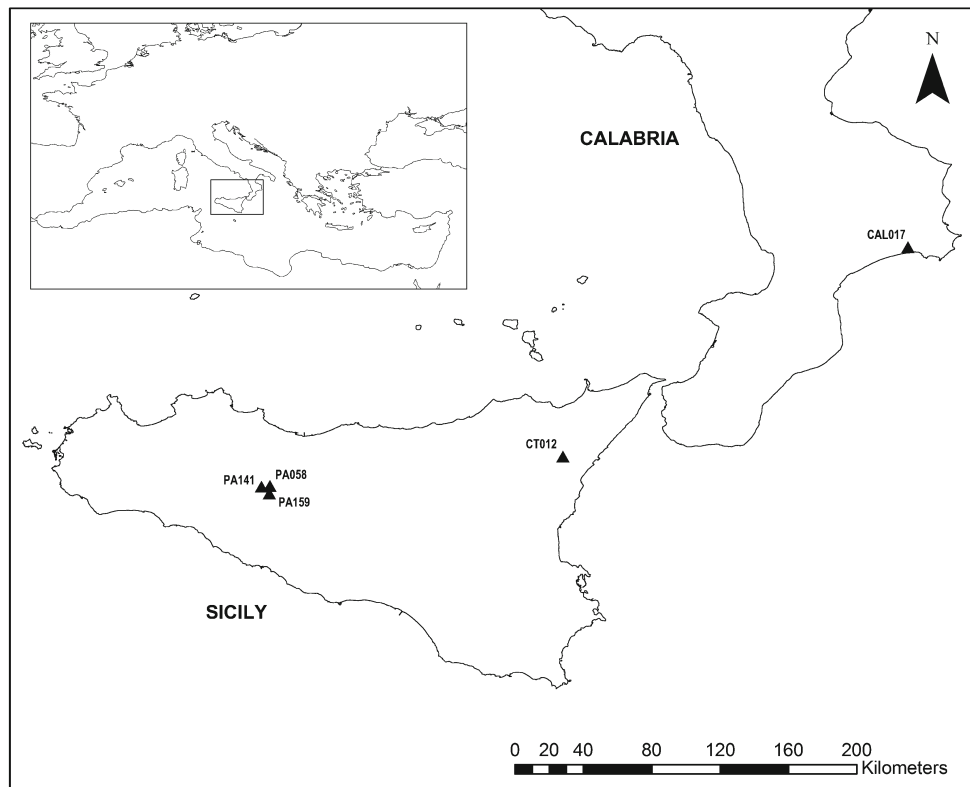


Fig. 1. Location of the *Ferrissia fragilis* occurrence sites in Sicily and Calabria. See Table 1 for the codes of the sites.

autochthonous in the western Palaearctic area. Such a late 'discovery' of a freshwater limpet which proved to be widespread in the whole Europe was explained either considering it an indigenous and overlooked taxon previously mis-identified with the apparently similar acroloxid limpet *Acroloxus lacustris* (L., 1758), or considering it a recent invader arrived in Europe either from North America, Northern Africa or Asia.

In Italy, the first representatives of the genus were recorded in the late 50s in the Lake Mergozzo and in the neighbouring Lake Maggiore. These specimens were used for the original description of "*Watsonula wautieri*" (now *Ferrissia wautieri*) (Mirolli 1960). Its currently known distribution range in Italy includes most of the Italian peninsula and Sardinia (Cianfanelli et al. 2007) while it was never reported to date for the southernmost part of the country, i.e., Sicily and Calabria. Although it is considered a non-indigenous species, current Italian literature keeps on using the binomen *F. wautieri* due to persisting doubts about the identity of southern European populations (Cianfanelli et al. 2007). In fact, Falkner & Proschwitz (1998) and Walther et al. (2006c) stressed the possibility that some autochthonous populations of *F. clessiniana* may occur in Southern Europe and North Africa.

An argument against the possibility that the European *Ferrissia* belong to an indigenous and till-recently-overlooked taxon is that it would imply the misidentification of the ancyloid genus *Ferrissia* with the acroloxid genus *Acroloxus*. This, although possible (e.g., see discussion in Walther et al. 2006b), seems quite unlikely as it should have affected all the malacological stud-

ies in Europe till the middle of the twentieth century; furthermore, it seems that no *Ferrissia* specimens are present in European malacological collections older than the twentieth century. Conversely, the hypothesis that the *Ferrissia* species occurring in Europe is an allochthonous taxon currently expanding its range through the whole continent is also supported by the increasing number of records reporting the species for most European countries in the last few years (e.g., Falkner & Proschwitz 1998; Altaba 2007; Son 2007; Beran & Horsák 2007; Semenchenko & Laenko 2008; see Bank 2010 for a summary), and from some recent molecular evidences (Walther et al. 2006b, c; present study).

Material and methods

Ferrissia specimens were collected in the frame of a wider survey aimed at the census of Southern Italian freshwater invertebrates. Visited sites were sampled both visually and through the use of 200 µm mesh-sized hand nets passed through the vegetation and on the bottom of the water bodies. Collected samples were fixed *in situ* in 80% ethanol and sorted in laboratory under a stereomicroscope. *Ferrissia* specimens were then sorted out and stored in separate vials in 95% ethanol at 4°C. Out of 596 sampled water bodies (554 in Sicily and 42 in southern Calabria), the occurrence of *Ferrissia* sp. was registered in four sites in Sicily and a single site in Calabria (Fig. 1). Table 1 summarizes the main characteristics of the collection sites, these are lentic water bodies fed by small springs, characterized by pronounced seasonal water level fluctuations. In all the sites, *Ferrissia*

Table 1. List of the *Ferrissia fragilis* occurrence sites in Southern Italy.

Code	Site Name	Region	Municipality	Sampling Date	Latitude (N)	Longitude (E)	Altitude (m a.s.l.)
PA058	Stagno di San Luca	Sicily	Castroville di Sicilia	20 XI 2009	37.7104	13.5726	750
PA141	Margio di Carcaciotto	Sicily	Castroville di Sicilia	15 IX 2009	37.7061	13.5283	967
PA159	Gorgo di S. Andrea	Sicily	Castroville di Sicilia	15 XII 2009	37.6709	13.5692	585
CT012	Stagno di C.da Pantano	Sicily	Castiglione di Sicilia	28 XII 2009	37.8641	15.1129	621
CAL017	Stagno di Steccato	Calabria	Cutro	17 III 2010	38.9647	16.9277	37

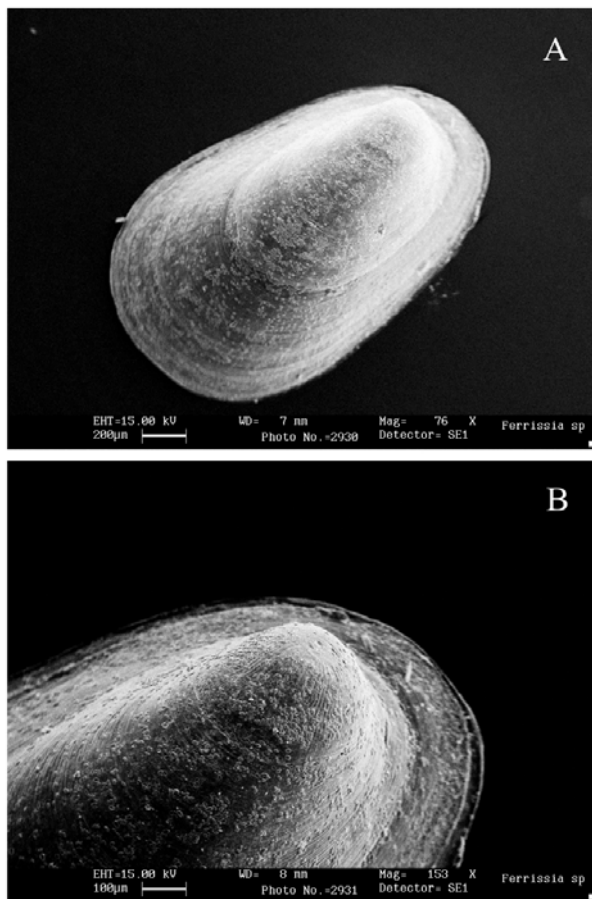
Fig. 2. *Ferrissia fragilis* from Margio di Carcaciotto (PA141, Sicily). A – Shell; B – Detail of the apex.

Table 2. Accession numbers of the sequences deposited in GenBank.

Code	16S	COI
PA058	HQ732263	
PA141	HQ732260, HQ732261	HQ732255
PA159	HQ732262	
CT012	HQ732259	HQ732256
CAL017	HQ732258	HQ732257

specimens were observed and collected on aquatic vegetation and decaying vegetal materials.

Scanning Electron Microscope (SEM) was used to identify the collected specimens (Fig. 2) but, in the light of the scarce discriminatory ability of morphology for the species of the genus, two mtDNA markers were also used

in order to solidly identify the Southern Italian specimens and to track their possible origin and dispersal pathways. Six specimens from five sites were included in the molecular analyses (Table 2). Prior to DNA extraction, specimens were carefully cleaned and soaked in double-distilled water for 3 h. DNA was then extracted using whole specimen and the “DNEasy Animal Tissue Kit” (Qiagen). A portion of the large ribosomal subunit 16S was amplified using primer sequences (5'- TGG TAA GGT AGC ATA ATA AT -3') and (5'- CCG GTT TGA ACT CAG ATC ATG T -3') (Thum & Harrison 2009); A partial sequence of the cytochrome oxidase 1 (COI) was amplified in three specimens using the universal primers LCO1490 (5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3') and HCO2198 (5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3') described by Folmer et al. (1994).

For the amplification of the 16S, PCR mix consisted of 3.5 µl of Buffer 10X (Thermoscientific), 3 µl of 25 mM MgCl₂, 0.35 µl of each dNTP 10 mM, 1 µl of each 10 µM primer, 0.4 µl of Red Hot Taq Polymerase 5U/µl (Thermoscientific), 1 µl of DNA template, and 23.7 µl of double-distilled water, for a total reaction volume of 35 µl. The amplification consisted of an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 50 s, 42°C for 50 s, and 72°C for 1 min, followed by a final extension at 72°C for 8 min. For the amplification of the COI, PCR mix consisted of 3.5 µl of Buffer 10X (Thermoscientific), 4 µl of 25 mM MgCl₂, 0.5 µl of each dNTP 10 mM, 0.36 µl of each 50 µM primer, 0.4 µl of Red Hot Taq Polymerase 5U/µl (Thermoscientific), 1 µl of DNA template, and 23.4 µl of double-distilled water, for a total reaction volume of 35 µl. The amplification consisted of an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 50 s, 42°C for 50 s, and 72°C for 1 min, followed by a final extension at 72°C for 8 min.

To check for the quality of the PCR products, 5 µl were separated by electrophoresis on a 2% agarose gel at 70 V for 1 h and visualized with a UV Transilluminator. When amplified bands were sharp and clean, the whole PCR products were purified using the Exo-SAP purification kit and sequenced in forward direction with an Applied Biosystems 3730xl DNA sequencer. Chromatograms were imported and edited with Chromas Lite 2.01 (Technelysium Pty Ltd) and exported to be aligned with ClustalX (Thompson et al. 1997).

In order to compare the sequences obtained from the Southern Italian populations with those available for other countries, all the available COI and 16S *Ferrissia* spp. sequences were downloaded from GenBank, including some *Ancylus fluviatilis* Müller, 1774 sequences to be used as outgroups; the poor quality of the available COI sequence of *Ferrissia clessiniana* from Uganda (Accession Number: AY577509) and *Ferrissia rivularis* (Say, 1817) from Maryland (A.N.: AY282586) did not allow us to include them in the analysis.

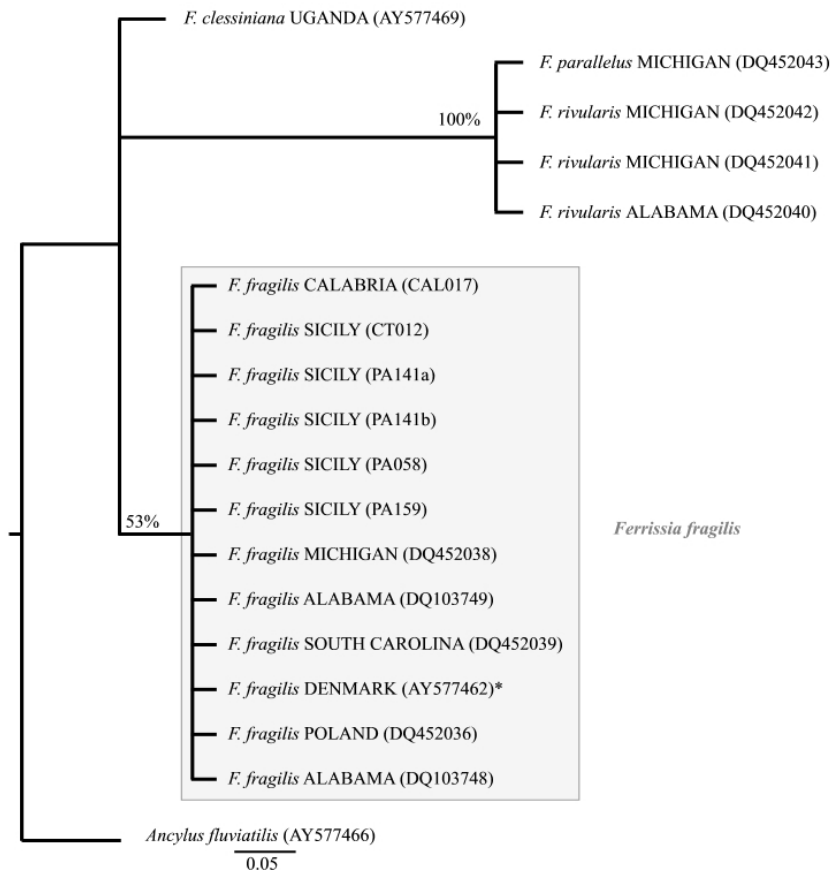


Fig. 3. Bayesian phylogram (ngen = 500,000; nchains = 4; nrun = 2; samplefreq = 100; temp = 0.2; default priors) of the mitochondrial large subunit ribosomal DNA (mtDNA 16S) dataset composed of *Ferrissia* spp. and the outgroup *Ancyclus fluviatilis*. Nodal posterior probabilities are given at the respective branches. GenBank accession numbers are given for non-novel haplotypes. * Mislabelled as *Acroloxus lacustris* (see discussion in Walther et al. 2006b).

The molecular identification of Southern Italian specimens and the reconstruction of the phylogenetic relationships among the taxa were carried out using Bayesian Inference as implemented in MrBayes v.3.2.1 (Ronquist & Huelsenbeck 2003). The best evolutionary model for each dataset was established by Akaike Information Criterion, performed with the software MrModelTest2.2 (Nylander 2004). For the COI dataset, Bayesian analyses was performed using a general time-reversible model of sequence evolution for molecular data with a proportion of invariable sites (GTR+I) (Prset statefreqpr = dirichlet (1,1,1,1); Lset nst = 6 rates = propinv). For the 16S dataset, the analyses was performed using a general time-reversible model of sequence evolution with gamma-distributed rate variation across sites (GTR+G) (Prset statefreqpr = dirichlet (1,1,1,1); Lset nst = 6 rates = gamma).

For both markers, two independent runs were performed with 500,000 generations and four Markov chains using default heating values. Trees and parameter values were sampled every 100 generations resulting in 5,000 saved trees per analysis; in both the analyses convergence was reached before the 50,000th generation, accordingly 500 trees were discarded as “burn-in”.

Results

The amplification of the 16S led to a 353 base-pairs long sequence, that of COI to a fragment 682 base-pairs long.

Their alignment with the sequences downloaded from GenBank did not evidence any gap or insertion and, after having trimmed the tails which were not present in all the individuals, led to a 16S aligned fragment of 321 base pairs, and a COI alignment of 655 base pairs.

For both genetic markers (Figs 3 and 4) Southern Italian *Ferrissia* specimens showed haplotypes which nested within the *F. fragilis* clade (including specimens from U.S.A., Northern Europe and Eastern Asia). Interestingly, a single 16S haplotype was observed in Southern Italy; this is identical to the haplotype observed in Poland (Accession Number: DQ452036), Michigan (A.N.: DQ452038), South Carolina (A.N.: DQ452039) and Alabama (A.N.: DQ103749) and differs only 0.3% (p-distance) from the Danish specimen (A.N.: AY577462) and 0.6 % (p-dist) from a second specimen from Alabama (A.N.: DQ103748). Unfortunately, no 16S sequences for the Asian *Ferrissia fragilis* specimens were available, but it is likely that the 16S haplotype recorded in Southern Italian samples is the commonest one in the allochthonous *Ferrissia* populations. Observed COI haplotypic diversity is slightly higher, with a single haplotype shared by the two Sicilian and the two Asian populations, and private ones (differing 0.2–0.7% in p-distance) for the populations from Calabria, Poland and the U.S.A.

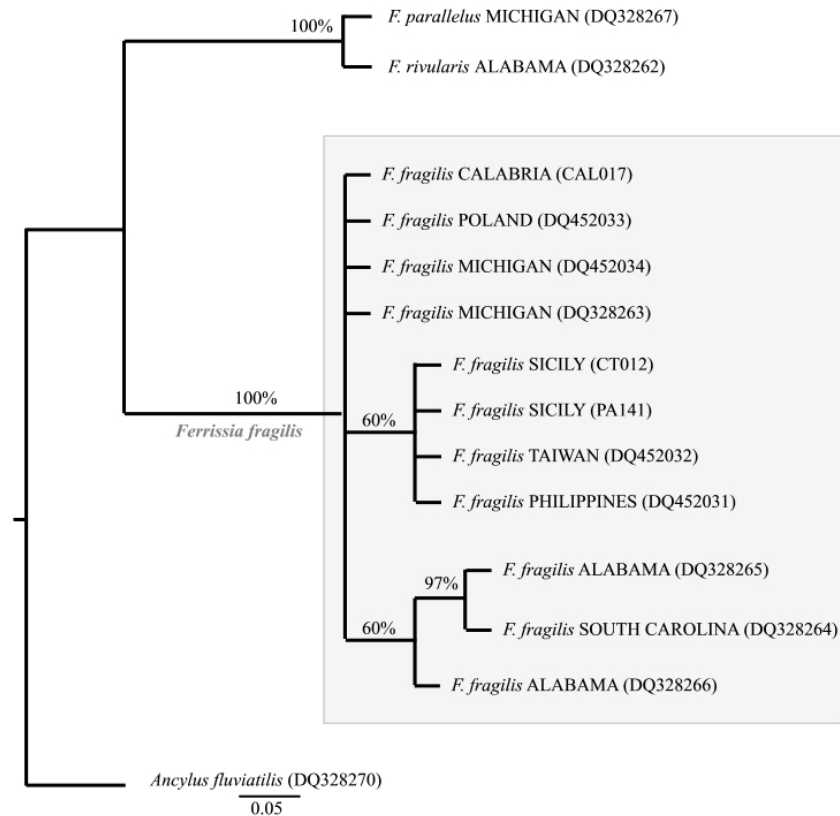


Fig. 4. Bayesian phylogram (ngen = 500,000; nchains = 4; nrun = 2; samplefreq = 100; temp = 0.2; default priors) of the cytochrome oxidase 1 (mtDNA COI) dataset composed of *Ferrissia* spp. and the outgroup *Ancylus fluviatilis*. Nodal posterior probabilities are given at the respective branches. GenBank accession numbers are given for non-novel haplotypes.

Discussion

Used molecular markers identified unequivocally the *Ferrissia* specimens from Sicily and Calabria as *Ferrissia fragilis*; the topologies of the trees obtained with both 16S and COI stress the molecular homogeneity of the European and Asian *F. fragilis* samples, and their identity with some of the *F. fragilis* haplotypes recorded in North America (Figs 3 and 4). In particular, the presence in Southern Italy of a single 16S haplotype of the North American species *F. fragilis*, which is also shared with the two eastern Asian *Ferrissia* populations, and the modest haplotypic diversity registered for the COI strongly support the hypothesis of a recent invasion of the Palaearctic region by a single strain of *F. fragilis* coming from the U.S.A. Thus, in spite of the finding of some Plio-Pleistocene fossils of the genus *Ferrissia* in Europe (van der Velde 1991), current European and Asian populations of *Ferrissia* seems to be constituted by the invasive American species *F. fragilis*, the protagonist of an overlooked biological invasion which is currently underway.

The absence of populations of an autochthonous Palaearctic *Ferrissia* species in Southern Italy casts some doubts on the hypothesis that indigenous *Ferrissia* species may actually occur in Europe (cf. Falkner & Proschwitz 1998; Walther et al. 2006c); however, in order to clarify this point, it is important to include in the molecular analyses some specimens from other

southern European countries, Middle East and Africa. The actual existence of *F. wautieri* and *F. clessiniana* itself needs to be tested, studying with molecular tools topotypical specimens.

In the light of the scarce molecular diversity observed in the whole Eurasia and of the growing evidences for the ongoing spreading of the species in Europe, the occurrence of a single invasion event from North America, or of repeated introductions from a single area of origin, is suggested. The arrival of *F. fragilis* in Europe is likely to be ascribed to the unware conveyance of the species along with the aquarium plant trade, followed by the involuntary release of the species by aquarists along with aquarium waste disposal, e.g., Duggan (2010) reports the finding of live *Ferrissia* sp. specimens in the sediment of aquariums in New Zealand. This hypothesis is also supported by the low genetic diversity of the introduced populations, a pattern which is typical for the biological invasions linked with aquarium trade (Roman & Darling 2007) and which is known not to affect the success of the invasion (Golani et al. 2007). After the first releases, the species may have independently spread through human activities and avian vectors.

The history of the invasion of *F. fragilis* in Europe faithfully follows that of another North-American freshwater gastropod, the physid *Haitia acuta* (previously named as *Physa acuta*, *Physella acuta*), which successfully invaded nearly all the freshwater ecosystems of the

world in the last two centuries (Dillon et al. 2002; Taylor 2003; Bousset et al. 2004; Wethington & Lydeard 2007) and that was initially considered an indigenous European taxon, described under the name of *Physa pisana* (Issel, 1868). The introduction and spreading of *H. acuta* in Europe is one of the causes of the progressive decline of the indigenous basommatophore *Physa fontinalis* (L., 1758) (e.g., Manganelli et al. 2000; Cianfanelli et al. 2007), and its invasion in other continents is currently monitored with great concern (e.g., Albrecht et al. 2009). No data are currently available on the possible impact of *F. fragilis* on the autochthonous molluscan communities, and especially on the autochthonous ancyliid *Ancylus* sp. with which the species often co-exist; the lack of precise data pre-dating the invasion of this allochthonous limpet prevents from getting a clear frame of its impact in Southern Italy; the realization of careful monitoring programs throughout its invasive range is thus advisable.

Despite the attention that biological invasions have received in the last years, present record of several well-established populations of an invasive species in the water bodies of Southern Italy stresses the hiatus currently affecting our knowledge of several "less charismatic" invertebrate groups in wide geographic areas, and the advisability of the realization of extensive and accurate faunal surveys in these areas. Furthermore, especially for those taxa affected by poorly resolved taxonomies, exotic species may go unnoticed or confused with indigenous ones: the invasion of *F. fragilis* in Europe and Asia has been overlooked for some decades, thus being largely cryptic, and only the recent molecular studies proved to be able to enlighten it. In such cases, molecular analyses prove to be a sound and reliable tool in order to unequivocally identify possible allochthonous species (e.g., Genner et al. 2004; Walther et al. 2006c; Miglietta & Lessios 2009) and to timely monitor the invasions.

Acknowledgements

The support of A.M. Mannino (Università di Palermo, Italy) with the Scanning Electron Microscope, and of G. Alfonso (Università del Salento, Italy) in the frame of the field surveys is gratefully acknowledged. Research partially funded by the Sicilian "Assessorato Regionale Territorio ed Ambiente" in the frame of the project "Censimento e valutazione della biodiversità degli ecosistemi acquatici delle R.N.O. 'Monte Genuardo e Santa Maria del Bosco', 'Monte Carcaci' e 'Monte Cammarata' all'interno dell'istituendo Parco Regionale dei Monti Sicani".

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Received July 16, 2010

Accepted December 30, 2010