

## Research Article

## *Ferrissia fragilis* (Tryon, 1863): a freshwater snail cryptic invader in Brazil revealed by morphological and molecular data

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### Abstract

The results of our study confirm the occurrence of the cryptic invader *Ferrissia fragilis* (Tryon, 1863) in Brazil, a species of worldwide geographical distribution and with poorly known morphology that is pervasive in several countries. Specimens were collected in the states of Rio de Janeiro and Minas Gerais in southeastern Brazil. We describe their morphology, and analyze the similarity of haplotypes generated from these samples with those previously obtained for *F. fragilis*. Shell morphology was compared by light and scanning microscopy. Soft parts of stained dissected specimens were studied under the stereomicroscope. Molecular analysis was performed on three specimens from each sample using the mitochondrial cytochrome c oxidase I gene. Based on a comprehensive analysis, including both morphological and molecular methodologies, we were able to identify the examined specimens as *F. fragilis*.

**Key words:** Ancyliinae, biological invasion, cytochrome c oxidase I, freshwater snail

### Introduction

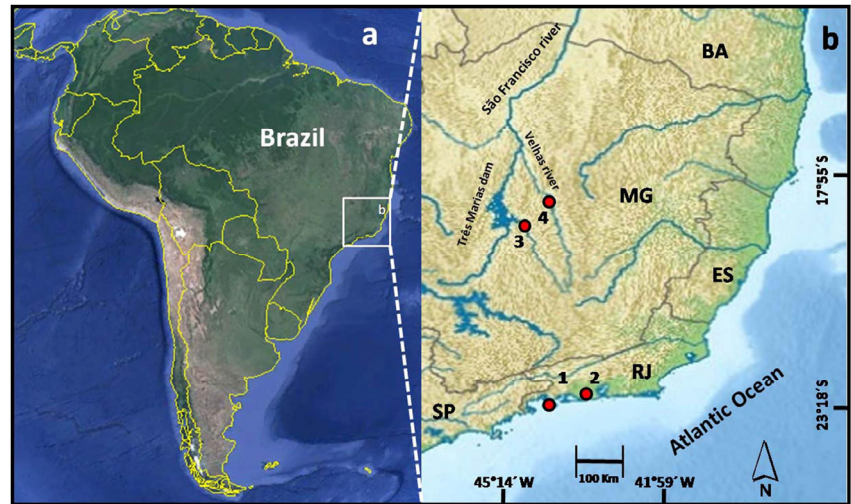
The introduction of non-native species is considered a main cause of biodiversity loss worldwide (Clavero and García-Berthou 2005). In limnic ecosystems, exotic species can cause deep changes in community structure, leading to the decline of local populations or even to their extinction (Kaufman 1992; Vitousek et al. 1997; Lydeard et al. 2004; Son et al. 2007), in addition to causing serious economic damage (Strong et al. 2008; Santos et al. 2012). In South America, some exotic molluscs have received more attention due to of the obvious problems they cause (Santos et al. 2012), for example the thiarid *Melanoides tuberculata* (Müller, 1774) and the limnic clams *Limnoperna fortunei* (Dunker, 1857), *Corbicula fluminea* (Müller, 1774) and *Corbicula largillierii* (Philippi, 1844), others were not recognized as non-native for decades. In the

emblematic case of *Physa acuta* (Draparnaud, 1805), this freshwater mollusc probably originating from North America and invasive in four continents, was described as a new species *Physa cubensis* Pfeiffer, 1839 native to the Neotropical region. The phenotypic plasticity of the shell has led to a list of more than 100 taxa (Paraense 2011) that are recognized as synonyms by biological (Dillon et al. 2002), morphological and molecular studies (Paraense and Pointier 2003).

*Ferrissia fragilis* (Tryon, 1863) of the sub-family Ancyliinae presents a similar case in Europe and Asia. This North American freshwater limpet was given different names by different researchers (Beran and Horsák 2007; Marrone et al. 2011), and is now considered a cryptic invader (Walther et al. 2006; Marrone et al. 2014; Albrecht et al. 2014).

*Ferrissia* (Walker, 1903) is characterized by the presence of radial lines at the apex of the

**Figure 1.** Maps showing the localities where *Ferrissia fragilis* was recorded. a- South America. b- Location of the four sampling sites: 1- Vila do Abraão stream, Municipality of Angra dos Reis, state of Rio de Janeiro (RJ); 2- Mucuíba waterfall, Municipality of Rio de Janeiro, state of Rio de Janeiro (RJ); 3-Três Marias Dam, Municipality of Felixlândia, state of Minas Gerais (MG) and 4- Velhas river, Municipality of Lassance, state of Minas Gerais (MG).



embryonic shell (protoconch) (Walker 1903; Hubendick 1964; Lanzer 1996; Santos 2003; Ovando et al. 2014). It has the widest geographical distribution among freshwater limpets, with records from different regions; North America (Walker 1903; Hubendick 1964, 1967; Walther et al. 2006), Europe (Son 2007; Marrone et al. 2011), Africa (Walker 1923), France (Wautier et al. 1966) and Australia (Hubendick 1967). Albrecht et al. (2014) is the most recent global phylogeny on *Ferrissia*, with special reference to *F. wautieri* (Mirolli, 1960) specimens collected at the type locality (Lake Mergozzo, Verbano-Cucio-Ossola, Italy); these sequences were included and discussed in this paper.

In South America, two species of *Ferrissia* were detected, *F. irrorata* (Guilding, 1828) has been recorded in Colombia (Gómez et al. 2004) and Argentina (Ovando et al. 2014) and *F. gentilii* Lanzer, 1991 in southern Brazil (Lanzer 1991) as the first species of the genus described for that region. There are records of *Ferrissia* sp. for different localities in southeast Brazil (Thiengo et al. 2001; Santos et al. 2003, 2012) but the identifications require confirmation.

Molecular methods have become a useful tool for species identification when morphological information is not informative, contributing to the advancement of taxonomy (Arif and Khan 2009). The use of the DNA barcoding technique as a tool for species determination, mainly due to its contribution to standardization and data validation (Romero and Ramírez 2011), and as a fast and low-cost method of identification (Golding et al. 2009), is quite appropriate in this situation.

Considering ancylids, most species diagnoses are based only on continuous morphological data of the shell, such as the position of the apex (Walther et al. 2010) and the width/length ratio, among other characters that may depend on environmental influences. In the case of cryptic species, which are morphologically indistinguishable, the use of this tool is indispensable. It is especially helpful in the case of *Ferrissia* species, where the small size hampers morphological studies.

The objective of this study was to integrate morphological and molecular information about representatives of *Ferrissia* from different populations in two Brazilian states, and to compare their DNA sequences with that of *F. fragilis*, a cryptic invasive species in several countries, aiming to correctly identify *Ferrissia* species on Brazilian territory.

## Methods

Samples were collected from four localities: 1) a stream in Vila do Abraão, Ilha Grande, Municipality of Angra dos Reis, state of Rio de Janeiro; 2) a waterfall in Mucuíba, Vargem Grande, Municipality of Rio de Janeiro, state of Rio de Janeiro; 3) the Velhas river, Municipality of Lassance, state of Minas Gerais; 4) the Três Marias Dam, Municipality of Felixlândia, state of Minas Gerais (Figure 1).

The Vila do Abraão stream has been anthropogenically impacted by riverbank modifications (Figure 2a), domestic sewage disposal influent and introduction of various limnic gastropods

over time (Santos et al. 2007, 2010; Miyahira et al. 2010; Lacerda et al. 2011; Gonçalves et al. 2014).

The Mucuíba waterfall (Figure 2b) is located in a tourist region, on the edge of the Pedra Branca State Park (PEPB). Although a recreation site, it is well-preserved with riparian vegetation and is unpolluted by domestic sewage. Both locations are located in remnants of the Atlantic Forest, a priority area for conservation (Myers et al. 2000).

The Velhas river (Lassance) and the Três Marias dam (Felixlândia) are part of the São Francisco river watershed. These localities represent the two major affluents of the high São Francisco basin (Pereira et al. 2007), the Velhas river and Paraopeba river, which empty into the Três Marias dam (Figure 2c). While the sampled stretch of the Velhas river is located in an inhabited area and is characterized by riparian vegetation (Figure 2d), the Três Marias Dam locality has no riparian vegetation or nearby buildings. *Eichhornia* sp., an invasive aquatic plant that can influence the distribution and richness of various macroinvertebrates (Henry-Silva et al. 2010) was present at both locations. Substrates were sandy, except that at the Três Marias dam (Felixlândia) which was clay.

Specimens were collected manually from the underside of decayed leaves, branches and stones, near the margins of waterbodies. Sample processing followed Santos (2003) and Lacerda et al. (2011, 2013). Fieldwork was conducted under legal authorization (SISBIO 10812-1 and 23607-2). The studied specimens are housed at the Malacological Collection of the Universidade do Estado do Rio de Janeiro (Col.Mol. UERJ).

Examined material: Brazil, Minas Gerais: Felixlândia (Três Marias Dam, -18.79800°S, -44.95115°W), six animals (shells and soft parts), collected on 19 July 2013, Coll. L.E.M. Lacerda, (Col.Mol. UERJ 10420); Lassance (Velhas River, -17.97199°S, -44.53364°W), 141 animals (shells and soft parts), collected on 20 July 2013, Coll. L.E.M. Lacerda, I.C.B. Gonçalves and R.F. Ximenes, (Col.Mol. UERJ 10417). Rio de Janeiro: Rio de Janeiro (Mucuíba waterfall, Vargem Grande, -22.956973°S, -43.49040°W), five animals (shells and soft parts), collected on 25 July 2012, Coll. L.E.M. Lacerda, C.A.M. Alcântara and G.K.M. Nunes, (Col.Mol. UERJ 10403); Angra dos Reis (Vila do Abraão stream, Ilha Grande, -23.141778°S, -44.170056°W), 16 animals (shells and soft parts), collected on 5 July 2012, Coll. I.C.B. Gonçalves and R.F. Ximenes, (Col.Mol. UERJ 10402).

### *Morphological analysis*

Morphological identification was carried out based on the original descriptions and additional literature (Hubendick 1964, Lanzer 1991, Ohlweiler and Lanzer 1993; Santos 2003; Walther et al. 2010) with regard to shells and soft parts. Shells were drawn using a camera lucida; the position of the apex in relation to the shell antero-posterior axis was marked. Measurements of shells were obtained under a dissecting microscope using a caliper to the nearest 0.05 mm: length (L), height (H), anterior width (W1) and posterior width (W2) and five morphometric indices of shell shape: W1/L, W2/L, H/L, H/W1 and W2/W1; following Lacerda et al. (2011). Gold-coated specimens from lots 10402 and 10403 were studied by Scanning Electron Microscopy (SEM). The morphology of the muscle scar was evaluated after immersion of specimens in Lugol's solution. Radulae were extracted from the buccal mass using a KOH 5% solution, washed abundantly in water, mounted on a stub, coated with gold and scanned with a Carl Zeiss LEO 1450VP.

### *Molecular analysis*

Specimens were preserved in absolute ethyl alcohol (100%) and frozen at -15°C. DNA was extracted from foot-muscle tissue of three specimens of each population with the Qiagen QIAamp DNA FFPE Tissue kit. We amplified the mitochondrial cytochrome *c* oxidase I (COI) marker using the primers LCO1490 and HC02198 of Folmer et al. (1994). The PCR protocol was as follows: denaturation at 94°C for 2.5 min, followed by 40 cycles of 30 sec at 90°C, 1 min at 48°C with a temperature decrease of 0.3°C.s<sup>-1</sup> and 1 min at 72°C, and a final extension step for 3 min at 72°C (slightly modified from Folmer et al. 1994). The quality of the amplification product was examined by electrophoresis on a 2% agarose gel in 1x TAE. Sequencing was performed using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Inc.), with 25 cycles of 95°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The sequencing products were processed by an automated sequencer ABI 3500 capillary system (Applied Biosystems, Inc.).

Sequences were checked and aligned with Clustal W Multiple Alignment application (Thompson et al. 1997) as implemented in BioEdit (Version 7.1.3.0), verified by visual inspection and edited, creating a consensus sequence from



**Figure 2.** Several distinct environments where *Ferrissia fragilis* was recorded. **a-** Vila do Abraão stream, an altered environment with modified margins, **b-** Mucuiba waterfall, a preserved environment with riparian vegetation and submerged leaves to which individuals were attached, **c-** Três Marias dam, with artificial substrates that promote dispersal. **d-** Velhas stream, showing two collectors searching for specimens attached to macrophyte leaves. Photos by L.E.M. Lacerda.

forward and reverse reads. All determined sequences were entered into a BOLD database (<http://www.barcodinglife.com>) under the project acronym CSRM. We used Neighbor-joining (NJ) with the software MEGA (Version 5.20) (Tamura et al. 2011), based on the Kimura 2-parameter (K2P) model to calculate genetic distance and levels of genetic divergence. Among genotypes, and performed a Maximum Likelihood analysis (ML) to determine the GTR+G as the most appropriate model of sequence evolution based on the Akaike criterion (AIC).

We used various COI sequences of *Ferrissia fragilis*, *F. rivularis* (Say, 1817) and *F. parallela* (Haldeman, 1841) available in GenBank. Sequences of various species of the genera *Ancylus* (Müller, 1774) and *Biomphalaria* (Preston, 1910) were used as outgroups (Table 1). The choice of external group was based on the close phylogenetic relationship among these taxa based on morphological (Hubendick 1964, 1967) and molecular data (Walther et al. 2006, 2010; Albrecht et al. 2007, 2014).

## Results

We report the presence of *Ferrissia fragilis* for the first time in two Brazilian states (Figure 1), based on morphological and molecular data.

### *Morphological analysis*

The shells of 61 specimens of the four examined populations are elliptical (Figure 3a); apex obtuse to the right of the midline, on the posterior third of the shell, slightly flexed to the right; the embryonic shell (protoconch) with radial lines that do not reach the apical depression, arranged closely together with a tendency to fork (Figure 3b); the shell sculpture surface (teleoconch) without well-marked radial lines, but some diffuse radial lines on the anterior slope. The color of the shells ranged between empire yellow (Figure 4) and yellow aniline, according to the color catalog of Ridgway (1912).

Morphometric ranges and basic descriptive statistics for each sample of *F. fragilis* are shown



**Table 1.** List of recently determined and previously published COI sequences obtained from GenBank, showing taxa, collection locality, the reference for sequence determination and the GenBank accession code.

species	locality	author	code
<i>Ferrissia fragilis</i>	Philippines	Walther et al. 2006	DQ452031
<i>Ferrissia fragilis</i>	Taiwan	Walther et al. 2006	DQ452032
<i>Ferrissia fragilis</i>	Poland	Walther et al. 2006	DQ452033
<i>Ferrissia fragilis</i>	Michigan, USA	Walther et al. 2006	DQ452034
<i>Ferrissia fragilis</i>	Michigan, USA	Walther et al. 2006	DQ328263
<i>Ferrissia fragilis</i>	South Carolina, USA	Walther et al. 2006	DQ328264
<i>Ferrissia fragilis</i>	Alabama, USA	Walther et al. 2006	DQ328265
<i>Ferrissia fragilis</i>	Alabama, USA	Walther et al. 2006	DQ328266
<i>Ferrissia fragilis</i>	Sicily, Italy	Marrone et al. 2011	HQ732255
<i>Ferrissia fragilis</i>	Sicily, Italy	Marrone et al. 2011	HQ732256
<i>Ferrissia fragilis</i>	Calabria, Italy	Marrone et al. 2011	HQ732257
<i>Ferrissia fragilis</i>	Progradeç, Albania	Albrecht et al. 2014	KF737917
<i>Ferrissia fragilis</i>	Epirus, Greece	Albrecht et al. 2014	KF737916
<i>Ferrissia fragilis</i>	WestGreece, Greece	Albrecht et al. 2014	KF737918
<i>Ferrissia fragilis</i>	Verbano-Cucio-Ossola, Italy	Albrecht et al. 2014	KF737919
<i>Ferrissia fragilis</i>	Verbano-Cucio-Ossola, Italy	Albrecht et al. 2014	KF737920
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224646
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224647
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224648
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224649
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224650
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224651
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224652
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224653
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224654
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224655
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224656
<i>Ferrissia fragilis</i>	Brazil	Present study	KM224657
<i>Ferrissia parallela</i>	Michigan, USA	Walther et al. 2006	DQ328267
<i>Ferrissia rivularis</i>	Alabama, USA	Walther et al. 2006	DQ328262
<i>Ferrissia rivularis</i>	Alabama, USA	Walther et al. 2010	GU391035
<i>Ferrissia rivularis</i>	Alabama, USA	Walther et al. 2010	GU391036
<i>Ferrissia rivularis</i>	Maryland, USA	Albrecht et al. 2014	KF737913
<i>Ferrissia rivularis</i>	Washington, USA	Albrecht et al. 2014	KF737914
<i>Ferrissia rivularis</i>	Washington, USA	Albrecht et al. 2014	KF737915
<i>Ancylus fluviatilis</i>	Ireland	Walther et al. 2006	DQ328270
<i>Ancylus scalariformis</i>	Macedonia	Albrecht et al. 2006	DQ301839
<i>Ancylus tapirulus</i>	Macedonia	Albrecht et al. 2006	DQ301837
<i>Biomphalaria glabrata</i>	Brazil	Martin et al. 1999	AF199092
<i>Biomphalaria tenagophila</i>	Brazil	Martin et al. 1999	AF199089
<i>Biomphalaria straminea</i>	Brazil	Martin et al. 1999	AF199084
<i>Biomphalaria peregrina</i>	Argentina	Standley et al. 2011	GU168593

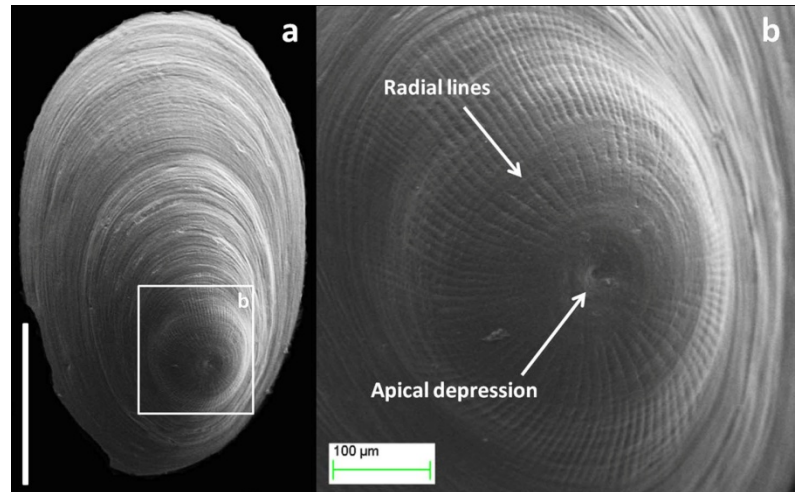
in Table 2. The overall means of measurements  $\pm$ SD (minimum-maximum ranges) in mm on all 56 shells, intact, were  $2.23\pm 0.34$  (1.38–3.01) length,  $1.38\pm 0.30$  (0.98–2.85) width and  $0.69\pm 0.12$  (0.34–0.94) height. The largest specimen (3.01mm length) was found in the Velhas river (municipality of Lassance), while the smallest (1.38 mm length) was found in the Vila do Abraão stream (municipality of Angra dos Reis, Ilha Grande).

All specimens have a darkly pigmented blotch concentrated on the anterior region of the mantle roof between the two anterior muscle scars, as well as scattered small blotches of dark pigmentation on the head (Figure 4a). The foot is rounded, never extending beyond the posterior shell

margin (Figure 4b). Mantle transparent, allowing the observation of internal organs; right anterior muscle scar elongated, transversely placed in relation to the median line of the body (Figure 4c). Short and rounded tentacles, with the basis tending to be triangular (Figure 4d), differing from those of other genera such as *Gundlachia* Pfeiffer, 1849, which have long tentacles (Lacerda et al. 2011, 2013), almost twice the length of *Ferrissia* tentacles.

The radula is a toothed chitinous ribbon responsible for rasping food. This structure contributes with a number of morphological characters used to diagnosis of species. The radular formula 13.1.13 express the number of tooth per row,

**Figure 3.** SEM images of the shell of *Ferrissia fragilis* (Tryon, 1863). **a-** Dorsal view, illustrating the absence of radial lines on the shell sculpture surface (teleoconch). Square: protoconch area. **b-** Detail of the embryonic shell (protoconch), showing the radial lines and apical depression. Scale = 1mm. Photo by A. Moraes for this study.



**Table 2.** Morphometric analysis of *Ferrissia fragilis* shells from four localities in Brazil. Means and standard deviation (mm), n = number of shells measured.

Morphometrical variables*	Vila do Abraão stream (n=15)	Mucuíba waterfall (n=5)	Velhas river (n=30)	Três Marias Dam (n=6)
L	1.98 ± 0.36	2.38 ± 0.44	2.38 ± 0.35	2.03 ± 0.24
W1	1.26 ± 0.21	1.41 ± 0.28	1.48 ± 0.34	1.19 ± 0.17
W2	1.09 ± 0.19	1.29 ± 0.26	1.21 ± 0.18	0.97 ± 0.12
H	0.57 ± 0.10	0.79 ± 0.11	0.74 ± 0.11	0.68 ± 0.08
W1/L	0.95 ± 0.05	0.59 ± 0.03	0.62 ± 0.07	0.58 ± 0.03
W2/L	0.55 ± 0.04	0.54 ± 0.02	0.51 ± 0.03	0.48 ± 0.03
H/L	0.29 ± 0.04	0.33 ± 0.02	0.31 ± 0.03	0.33 ± 0.02
H/W1	0.46 ± 0.07	0.57 ± 0.06	0.51 ± 0.06	0.57 ± 0.05
W2/W1	0.87 ± 0.04	0.92 ± 0.01	0.83 ± 0.07	0.82 ± 0.04

\*(L) length, (W1) anterior width, (W2) posterior width, (H) height, (W1/L) anterior width/length, (W2/L) posterior width/length, (H/L) height/length, (H/W1) height/anterior width, (W2/W1) posterior width/anterior width.

representing one central teeth flanked by 13 lateral and marginal tooth. Each central teeth of the radula has two symmetric median cusps (projections of the teeth with the same length) with a small accessory cusp between them and one or two cusps on each side of the main cusp (Figure 5a).

The lateral teeth are basically tricuspid, with the mesocon slightly more elongate, with a small cusp between the mesocon (intermediary cusp of lateral tooth) and the ectocon (the outermost cusp of lateral tooth) and two or three accessory cusps on the external side. Transition of lateral to marginal teeth is marked by decreasing size of teeth around the fifth to seventh teeth. The number of accessory cusps of tooth increases towards radular margin while size decreases (Figure 5b).

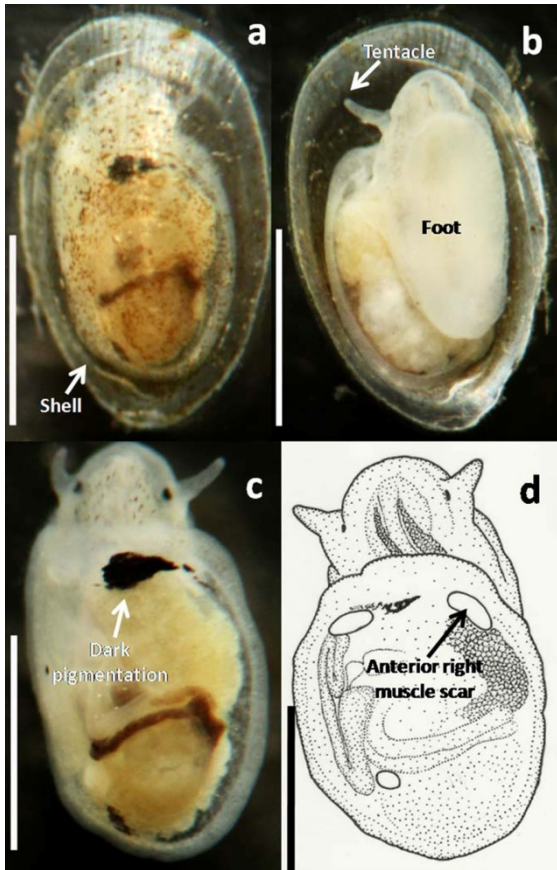
#### Molecular analysis

Twelve new COI sequences were generated and analyzed in this study. We obtained a database

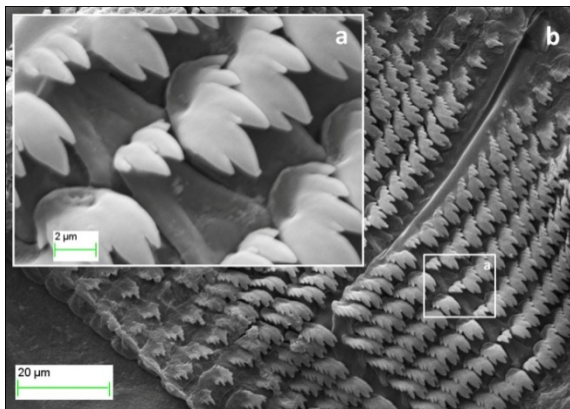
composed of 579 base pairs fragment of the COI mitochondrial marker (after editing) composed by both newly determined and previously published sequences.

The neighbor-joining (NJ) analysis is shown in Figure 6. Genetic distance (K2P) was 0.2–0.8% among *F. fragilis* haplotypes, 10.6–11.5% between *F. rivularis* and *F. fragilis*, 12.4–14.7% between *F. fragilis* and *Ancylus* spp. and 14.4–18.0% between *F. fragilis* and *Biomphalaria* spp. The two analyzes, NJ and ML, showed the same topology and high support values (Figure 6 and 7). The resulting tree recovered *F. fragilis* in a clade with 100% support, therefore suggesting *F. rivularis* / *parallela* as a sister clade (Figure 6). The *Ferrissia* Brazilian specimens formed two haplotypes, within a well-supported *Ferrissia fragilis* clade, together with representatives of different regions of the world (Figure 7).

Albrecht et al (2014) based on topotypes of *F. wautieri* (Table 1), concluded that the samples are genetically (COI) similar to *F. fragilis*. Our



**Figure 4.** *Ferrissia fragilis*. **a-** Dorsal view of the shell and soft parts. **b-** Ventral view of the shell and soft parts, showing the rounded shape of the foot and short tentacles. **c-** Soft parts, showing dark pigmentation only between the mantle scars. **d-** Drawing showing absence of mantle pigmentation and pattern of muscle scars; Scale = 1mm. Photos and drawing by L.E.M. Lacerda.



**Figure 5.** Radula of *Ferrissia fragilis*. **a-** Detail of the central tooth showing the two main cusps, the median accessory cusp and one or two external accessory cusps, at 10.570 x magnification. **b-** Dorsal view of radula, showing rows of teeth (Col.Mol. UERJ 10402), 5.000 x magnification. Photo: M.F. Oliveira for this study.

samples from Minas Gerais, provided a haplotype with a high support value (Figure 7), with the sequences obtained from specimens of the type locality of *F. wautieri*. So, our results corroborate the observation of Albrecht et al. (2014), that *F. wautieri* can be synonymized with *F. fragilis*. The other haplotype, from representatives of Rio de Janeiro and Angra dos Reis (Brazil), was grouped together with representatives from Philippines, Taiwan and Sicily.

A secondary analysis was performed with the insertion of the haplotypes of Walther et al. (2010) and Albrecht et al. (2014) (Supplementary material, Appendix 1). As observed by Walther et al. (2010), the Australian *Ferrissia* (*Pettancylus*) sp. is a sister group of *F. fragilis*, as previously suggested by Hubendick (1964) based on details of penis complex morphology.

## Discussion

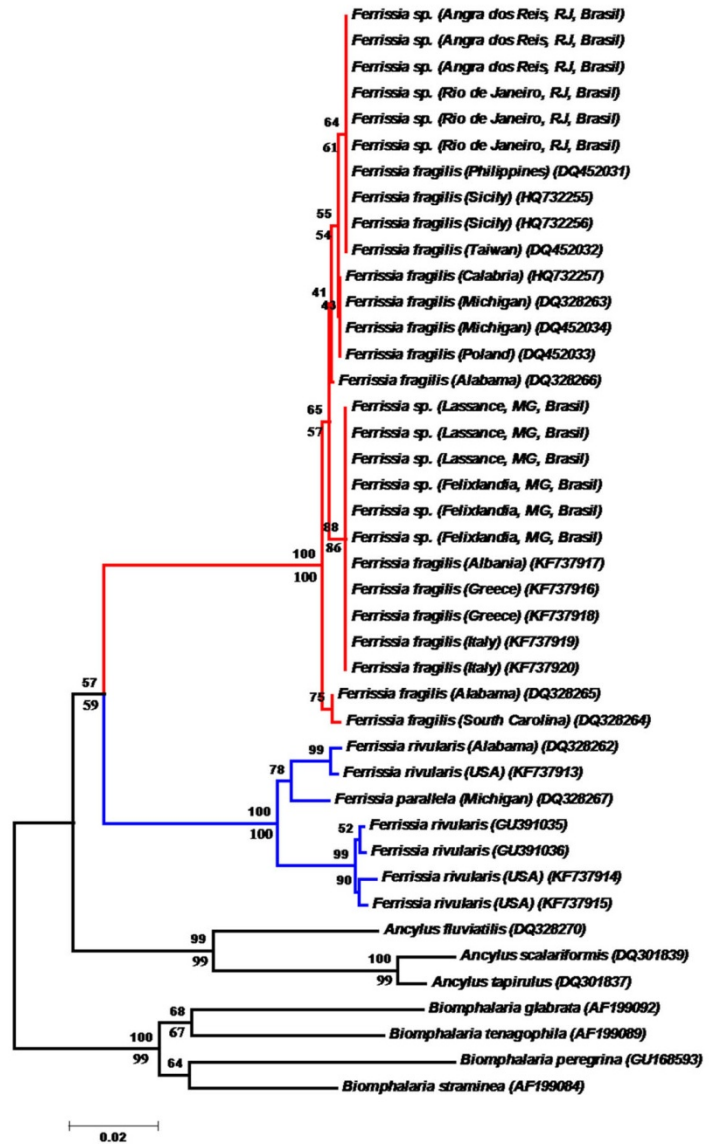
The holotype of *F. fragilis* (ANSP 22011) was damaged and did not allow comparisons with other material (Walther et al. 2010). In addition, the original species description is poor, based only on shell characters (Tryon 1863; Hubendick 1964), thus impeding morphological comparison. Several morphological and molecular studies performed with topotypic specimens of *Ferrissia* as *F. fragilis*; *F. rivularis* (Say, 1817); *F. parallela* (Haldemann, 1841); *F. walkeri* (Pilsbry and Ferriss, 1907); *F. sharpi* (Sykes, 1900); and *F. mcneilli* Walker, 1925 allowed synonymization of these species (Walther et al. 2010). *Ferrissia rivularis* and *F. fragilis* were considered valid species, being recognized by shell apex position and habitat.

*Ferrissia rivularis* has a more projected apex, near the shell median line, and prefers lotic habitats whereas *F. fragilis* has an apex positioned slightly to the right of the median line and prefers lentic habitats. Our results, based on molecular data, indicated the synonymy of *F. rivularis* and *F. parallela*, as was also observed by Walther et al. (2010).

The analysis of morphological characters (apex position, apical microsculpture, pigmentation of the mantle, shape of radula tooth) allowed us to identify the studied samples as *F. fragilis*, a result confirmed by molecular data.

*Ferrissia fragilis* is similar to *F. irrorata* in apex position and microsculpture, and both species have radial lines that do not reach the apical depression. Both differ from *F. gentilis* in southern Brazil, the radial lines of which reach the apical depression and are more delicate and

**Figure 6.** Neighbor-Joining tree based on the cytochrome c oxidase I (mtDNA COI) sequences of *Ferrissia* spp. (*F. fragilis* in red clade; *F. rivularis* in blue clade) and the outgroups *Ancyclus* spp. and *Biomphalaria* spp. Numbers above the branches represent bootstrap support values and numbers below the branches represent maximum likelihood. Accession numbers are given for haplotypes obtained from GenBank.



further apart, according to the illustrations of Lanzer (1991) and exam of the type material deposited in the Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre (Holotype MCN 31008-9).

Morphometric analysis of shells indicated their size to be smaller than 4 mm (Table 2), corroborating data from Walther et al. (2010).

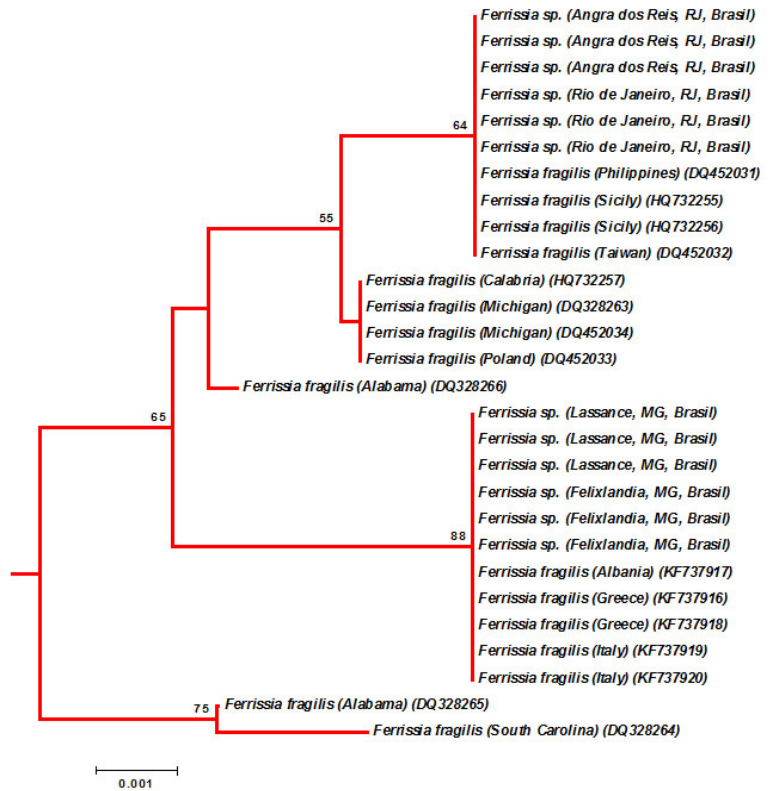
The studied specimens had a dark blotch of pigment between the two anterior muscle scars (Figure 4), similar to a specimen of *F. rivularis* (Say, 1817), the type-species of *Ferrissia*, illustrated by Hubendick (1964), but differing from *F. gentilis* in which the pigmentation is absent (Lanzer 1991).

The right anterior muscle and the posterior muscle scars are slightly more transversely elongated than the left anterior muscle scar (Hubendick 1964: Figure 70), similar to *F. gentilis*, as described by Lanzer (1991), and *F. irrorata* as described by Harrison (1983: fig 3b). Therefore, it seems that these characters are not suitable for species identification, although very useful to discriminate genera (Lanzer 1996; Santos 2003; Lacerda and Santos 2011; Ovando et al. 2014).

The central (rachidian) tooth of *F. fragilis* has two mean symmetric cuspids with a median accessory cusp, and one or two lateral small cusps, differing from the rachidian tooth of *F. gentilis*,



**Figure 7.** Neighbor-Joining phylogenetic tree of the freshwater snail *F. fragilis* and haplotype determination. Numbers above the branches represent bootstrap support values and numbers below the branches represent maximum likelihood (only those, higher than 50%). Accession numbers are given for haplotypes obtained from GenBank.



which has two symmetric median cusps, but with two or three median cusps and three to four lateral cusps (Lanzer 1991). Hubendick (1964), using a light microscope, observed the absence of a small median cusp between the main cusps of *F. rivularis*, and the presence of this cusp in *F. fragilis*. According to the illustrations of Harrison (1983), by SEM, it is not possible to see notable differences between the radula of the studied *F. fragilis* and *F. rivularis*, suggesting the need for comparative studies.

Harrison (1983) illustrated the central tooth of *F. irrorata*, which differs from *F. gentilis* by the presence of a median accessory cusp between the two main and the two lateral cusps on each side, differing from *F. fragilis*, which has one or two accessory cusps. On the other hand, the central tooth of *F. rivularis* illustrated by Hubendick (1964) has two symmetrical main cusps, with a median accessory cusp on each side and is distinguished from *F. fragilis* by the absence of an accessory cusp between the main cusps. We found no difference between the radulae of *F. fragilis* and *F. irrorata*. However, we recommend future comparative morphological studies of these two species.

Septate or gundlachioid shells have a horizontal septum reducing shell opening. It is formed when the snails face adverse environmental conditions, specially in times of drought (Mirolli 1960; Santos 2003; Ovando et al. 2011), preventing desiccation. Although observed for some populations of *F. fragilis* by Walther et al. (2010), no septate shells were found among the specimens of our study.

The genus *Ferrissia* was recovered as monophyletic within the Planorbidae, and sister to other members of the genus *Ancylus*. Walther et al. (2006) and Marrone et al. (2011) found the same relationships, based on nuclear (28S) and mitochondrial (COI) DNA, respectively.

The 12 new genotypes from specimens collected in two watersheds in Brazil (São Francisco basin and eastern Atlantic basin) are similar to samples of *F. fragilis* from various regions around the world (Albania, Greece, Sicily, Philippines, Taiwan, Poland and USA) (Figure 6 and 7). Although Figure 7 illustrates two distinct clades of *F. fragilis*, we found a short (0.08%) divergence between the analyzed sequences of the two Brazilian basins.

Nevertheless the haplotype formed with *F. fragilis* representatives found in the São Francisco

River Basin showed no genetic divergence between the sequences analyzed from Albania, Greece, and especially those of Italy (KF737919 and KF737920). These genotyped specimens of Italy were collected at the type locality of *F. wautieri* and fell to the same clade of *F. fragilis*, according to Albrecht et al. (2014). Thus, our results confirm that there is no divergence between the sequences of *F. fragilis* and *F. wautieri* based on COI gene. This may indicate that the group requires a taxonomic revision.

The South American continent has an interesting history of catchment formation, which underwent consecutive isolations and reconnections for millions of years, allowing for the diversification and endemism of different biota (Lundberg et al. 1998; Amaral et al. 2013). There are clear distinctions between origin and formation of the San Francisco basin, which is the oldest, and the Eastern Atlantic basin (Leal 2011).

Despite the historic diversification between the two basins, the specimens of *F. fragilis* analyzed in this study showed no significant molecular or morphological divergence. Based on these observations, we hypothesize that the current distribution of these four populations in the two basins could be due to cryptic introduction, as observed in other regions (Walther et al. 2006; Marrone et al. 2011, 2014; Son et al. 2007). Our hypothesis is supported by the presence of *Eichhornia* sp. in three localities (Lassance, Felixlândia and Angra dos Reis). This invasive plant causes changes in benthic communities of limnic ecosystems (Henry-Silva et al. 2010), including an increase of richness and dispersal of non-native molluscs (Santos et al. 2007; Miyahira et al. 2010; Gonçalves et al. 2014). The dispersal of *Ferrissia fragilis* by aquatic macrophytes is feasible due to its small body size and aestivation capability (Walther et al. 2006): during our collections we observed several specimens adhered to petioles close to the water surface, where their food (periphyton) is in good supply.

The two haplotypes observed in this study point to two possible independent events of biological invasion in Brazil so far. We believe that *F. fragilis* may have a wider distribution than that observed in the present study.

Walther et al. (2006) pointed out the importance of increasing our understanding of systematics, ecology and history of ancyliid invasions. The occurrence of *F. fragilis* has been reported from various regions around the world (Walther et al. 2006; Son 2007; Marrone et al. 2011, 2014;

Raposeiro et al. 2011), based mainly on molecular data. The present study aimed to add novel morphological information, such as scanning electron microscopy images of the radula, as well as molecular data.

## Conclusions

We report the occurrence of the cryptic invader *F. fragilis* in Brazil, based on analysis that included both morphological and molecular methods.

*Ferrissia fragilis* was identified by shell morphology (relative position of the apex and apical microsculpture) and soft parts (mantle pigmentation, shape of muscle scars and shape and number of cusps on the rachidian tooth). In addition, we confirm the occurrence of this cryptic invader in Brazil based on low intraspecific genetic divergence between haplotypes, which were analyzed for the first time in this country.

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The following supplementary material is available for this article:

**Appendix 1.** Neighbor-Joining tree based on the cytochrome c oxidase I (mtDNA COI) sequences of *Ferrissia* spp. (*F. fragilis* in red clade; *F. rivularis* in blue clade) and the outgroups *Ancylus* spp. and *Biomphalaria* spp.

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