Confirmation that the North American ancylid *Ferrissia fragilis* (Tryon, 1863) is a cryptic invader of European and East Asian freshwater ecosystems

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Freshwater biotas and ecosystems are being profoundly reshaped by ongoing human-mediated transoceanic biotic exchange.[1] Although the establishment of invasive freshwater mollusc populations may involve highly conspicuous ecological perturbations,[2,3] this process may also occur cryptically. Cryptic invasions are most likely to involve lineages with poorly resolved taxonomies and, in such cases, accurate diagnoses may require intercontinental genetic characterization and phylogenetic analyses.[4]

In a recent study,[5] we provided evidence that a published mitochondrial large ribosomal subunit (16S) genotype (GenBank number AY577462), sampled in Denmark and erroneously attributed to the Old World acroloxid endemic freshwater limpet *Acroloxus lacustris* (Linnaeus, 1758),[6] actually belonged to the North American ancylid freshwater limpet *Ferrissia fragilis* (Tryon, 1863). Although indirect, this was the first European record of *F. fragilis* since 1949, when a German sample of an enigmatic freshwater limpet was identified by J. Morrison as *F. shimekii* (Pilsbry),[7] a North American taxon later synonymized with *F. fragilis*.[8] The mystery European limpet had been discovered a few years previously in southern France,[9] and in the second half of the 20th century it was sequentially recorded throughout much of the continent under various names.[10–13]

Taxonomic uncertainty pervades much of the freshwater limpet literature, due in part to pronounced ecophenotypic plasticity in shell morphology.[8,14] A wide diversity of opinion, in addition to Morrison's,[7] has been expressed concerning the taxonomic and geographic affinities of the mystery European limpet species. It has been placed in assorted ancylid genera: *Gundlachia*, *Watsonula*, *Pettancylus* and *Ferrissia*;[10,15–17] described as a new species, *F. wautieri* (Mirolli, 1960);[18] and synonymized with the Near Eastern/African limpet *F. clessiniana* (Jickeli, 1882).[17] In the recent European literature it has been referred to as either *F. wautieri*[19,20] or as *F. clessiniana*,[13,21] and each name is charged with distinct biogeographic associations. *Ferrissia wautieri* is assumed to be endemic, and its absence from earlier European faunal surveys is attributed to its small size, formerly un-described status and misidentification as *Acroloxus lacustris*.[19,20] In contrast, *F. clessiniana* is assumed to have spread recently across Western/Central/Eastern Europe from presumed endemic foci in either Southern Europe and/or North Africa.[21]

Based collectively on the phylogenetic placement of a voucher-less GenBank sequence,[5] on Morrison's early (but overlooked) conchological identification,[7] and on a number of striking ecological, morphological and physiological similarities,[5] we proposed an alternate hypothesis: that the rapid expansion of this enigmatic limpet across European watersheds represents a cryptic invasion of New World *Ferrissia fragilis*.[5] This trans-Atlantic invasion hypothesis makes the explicit phylogenetic prediction that genotypes of verified European specimens will nest within a clade of North American *F. fragilis* and will be phylogenetically distinct from *Ferrissia clessiniana* genotypes sampled from its endemic range. Our goal in this study is to test the trans-Atlantic invasion hypothesis.

See Table 1 for sampling details. The mystery European *Ferrissia* was first recorded in Poland (identified as *F. wautieri*) in 1986.[22] In 2005, we obtained specimens sampled from ponds in Silesia, southern Poland (identified as *F. clessiniana*),[13] that we genotyped for one nuclear [784 nt aligned length of large nuclear ribosomal subunit (28S)] and two mitochondrial [670 and 443 nt respective aligned lengths of cytochrome oxidase subunit 1 (COI) and large mitochondrial ribosomal subunit(16S)] markers. These genotypes were phylogenetically analysed together with homologous sequences from North American *F. fragilis*, Asian samples of the *Ferrissia* subgenus *Pettancylus* (diagnosed primarily on geographic grounds[16]) and available GenBank genotypes from other ancylid taxa. Molecular and phylogenetic techniques employed to amplify, sequence and analyse the target gene fragments are detailed in two recent publications,[23,24] and novel sequences have been deposited in GenBank (28S: DQ452044–DQ452048; COI: DQ452031–DQ452035; 16S: DQ452036–DQ452043).

Figure 1 shows the gene tree topologies generated for the nuclear marker and mitochondrial COI datasets. A detailed discussion of those general aspects of the gene tree topologies not entertained here is available in a separate publication on ancylid molecular systematics.[23] For both genetic markers, the Polish specimens yielded genotypes that formed a clade together with those of North American *Ferrissia fragilis*, Taiwanese nominal *Pettancylus* and a subsample of Philippine nominal *Pettancylus* (Fig. 1). These globally distributed freshwater limpet specimens shared a single 28S genotype and displayed surprisingly modest levels of COI haplotypic diversity ($\leq$7 inferred mutational steps), most of which occurred among our North American samples. Remarkably, the four Polish specimens genotyped shared the modal (9/10) Michigan COI haplotype, whereas the Taiwanese and Philippine specimens differed from the latter by a single inferred mutational step (Fig. 1). This phylogenetic result is consistent with the hypothesis that *F. fragilis* has established cryptic invasive populations, not only in Poland, but also in Taiwan and the Philippines. Corroborating evidence is found in the presence of shared conchological features, diagnostic for *F. fragilis*,[8] in these far-flung populations (Fig. 1). These include a very small ($\leq$4 mm) and fragile shell

Table 1. Sampling locations for genotyped Polish and Asian limpets.

Nominal taxon	Collector	Locality	UMMZ catalogue number
Ferrissia clessiniana (Jickeli, 1882)	Strzelec	Upper Silesia, southern Poland	300279
Pettancylus sp.	Remigio	Alimodian, Iloilo Prov., Panay I., Philippines	300276, 300277
Pettancylus sp.	Wu	Chi-Chi, Nantou Co., Taiwan	300278

Taxonomic designations assigned by the collectors.

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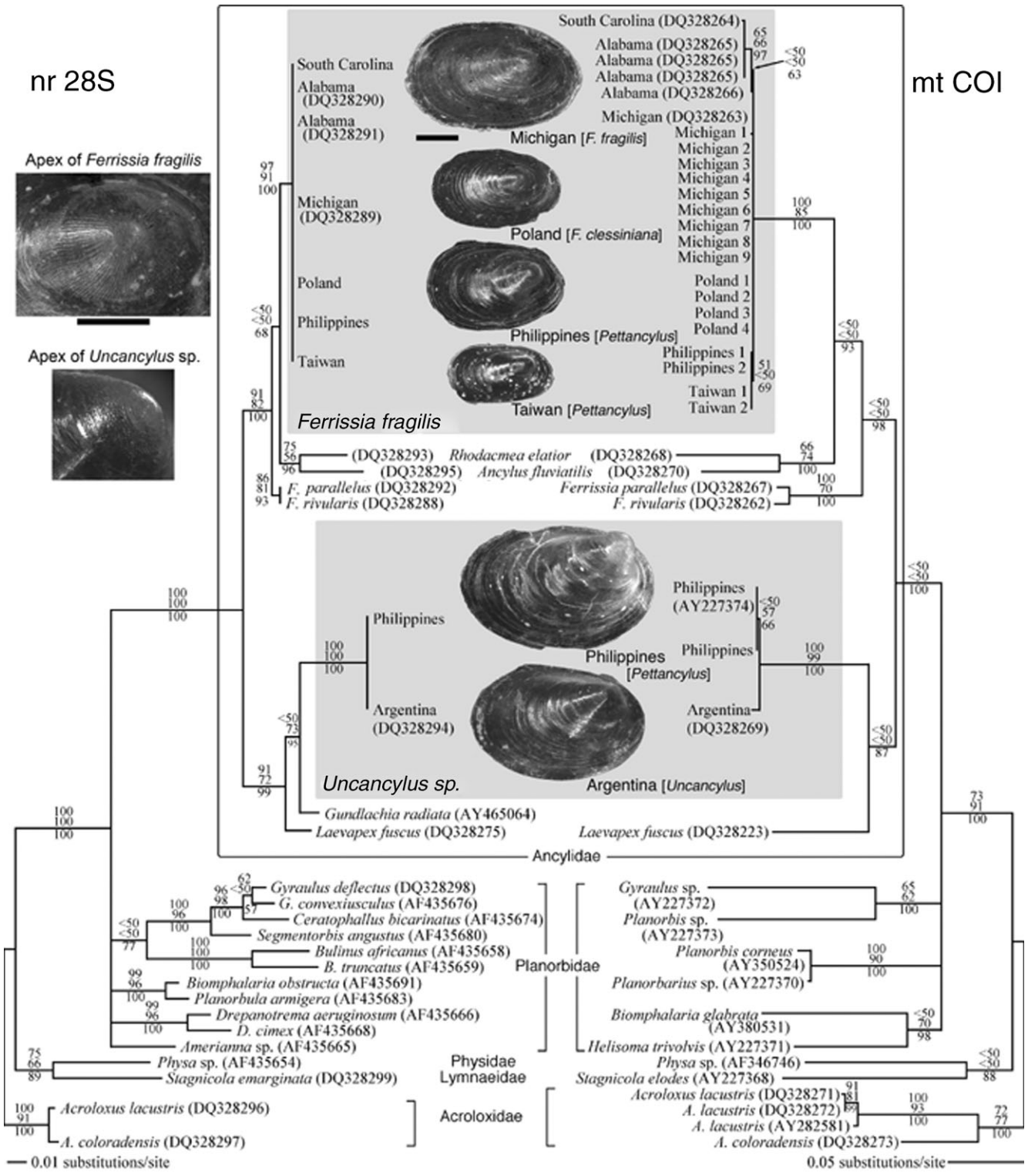

Figure 1. Bayesian consensus phylograms for nuclear large subunit ribosomal DNA (nr 28S) and mitochondrial cytochrome oxidase 1 (mt COI) datasets. Two *Acroloxus* species served as the designated outgroups, and nodal posterior probabilities are given below the respective branches. Above these branches, maximum parsimony (top) and maximum likelihood (middle) bootstrap support values are also provided. For non-novel genotypes, the GenBank accession numbers are indicated, including the *Uncancylus* mt COI (AY227374) previously obtained from the same Philippine location sample and misidentified as *Pettancylus*.[25] Dorsal view exemplar shell profiles (scale bar = 1 mm; original identifications provided in brackets) are shown for the Michigan, Polish, Philippine and Taiwanese samples of *Ferrissia fragilis* and the Philippine and Argentine samples of *Uncancylus* sp. Details of shell apex microsculpture are presented separately (scale bar = 0.25 mm) for a Polish *F. fragilis* specimen exhibiting the characteristic *Ferrissia* protoconch radial striations[8,26] and for a Philippine *Uncancylus* sp. specimen showing the microscopic punctate protoconch sculpture typical of this genus.[26]

with rounded ends, sides nearly parallel but diverging anteriorly, and a posteriorly-positioned apex that is elevated, acute and curved backwards with typical *Ferrissia* radial protoconch striations[8] that, as they diverge distally, accommodate new intervening striae.[26]

Although the Philippine sample of nominal *Pettancylus* specimens was obtained from a single sampling site (E. Remigio, personal communication), it contained two ancylid species. In addition to *Ferrissia fragilis*, the sample included specimens of a second, morphologically distinct ancylid limpet that yielded an identical 28S genotype, and a remarkably similar COI haplotype (four inferred mutational steps), to an Argentine sample of *Uncancylus* sp. (Fig. 1). This Philippine/Argentine molecular phylogenetic association was also corroborated conchologically: genotyped limpets belonging to the *Uncancylus* tip clade from either location displayed the diagnostic *Uncancylus* conchological features (Fig. 1) of a slender apex strongly hooked toward the right posterior side,[27] and a protoconch bearing a circular band of closely spaced microscopic shallow pits.[26] These results indicate that the Philippines have experienced not one but two cryptic invasions of phylogenetically distinct New World ancylid taxa.

Although we were unable confidently to assign a species-level identity, the Philippine *Uncancylus* specimens appear to represent only the second record of the ancylid subfamily Laevapecinae outside the New World. The first record concerned a new species, *Gundlachia hubendicki* (Brandt, 1974), with a type locality in Bangkok and a surprisingly restricted distribution within Thailand.[28] Brandt assumed it to be an endemic Asian laevapecinid; however, it is possible that his *G. hubendicki* may represent a separate invasive population of the *Uncancylus* taxon that we document in the Philippines. Additionally, Brandt's description and photograph of Thai specimens of another ancylid, *Ferrissia verruca* (Benson, 1855), are suspiciously similar to *F. fragilis*.[28] We do not have access at present to these Thai limpet populations; however, their specific status could be tested phylogenetically by genotyping specimens and determining if they respectively fall within the *Uncancylus* and *F. fragilis* clades (Fig. 1).

Our novel 28S and mt COI results (Fig. 1) fulfil the phylogenetic prediction of the trans-Atlantic European invasion hypothesis for *Ferrissia fragilis*,[5] at least for the sampled Polish population. However, the broader relevance of this result cannot be readily assessed with these markers due to the absence of genetic data for other European populations and also for non-European populations of *F. clessiniana*. Fortunately, supplementary mt 16S genotypes were available for single Danish[5,6] and German (C. Albrecht, unpublished) *Ferrissia* specimens and also for a Ugandan *F. clessiniana* specimen.[6] We genotyped our Polish and North American samples of *F. fragilis* for mt 16S and phylogenetically analysed them together with the supplementary sequences and a number of outgroup taxa (Fig. 2). The African *F. clessiniana* was sister to a robustly supported, phylogenetically shallow polytomy containing the North American *F. fragilis* and all three European genotypes independently sampled from Denmark, Germany and Poland (Fig. 2). The available data therefore corroborate the trans-Atlantic European invasion hypothesis for *F. fragilis*[5] and Morrison's initial identification,[7] although it is possible that phylogenetic characterization of southern European populations might eventually encounter *F. clessiniana* lineages.[21]

Our results indicate that multiple cryptic intercontinental invasions involving New World ancylid lineages are ongoing and that the North American species *Ferrissia fragilis* may be on its way to achieving a near-cosmopolitan distribution in temperate and tropical freshwater pond ecosystems. It is likely that basic life history attributes, including small body size, hermaphroditism, ability to live in stagnant water and ability to aestivate[8] underlay its pronounced invasiveness, though it is noteworthy that the first German,[7] Swedish[21] and British[12] records were from artificial habitats (aquaria and botanical gardens). The potential presence of multiple intercontinental alien cryptic invaders complicates the study of an already challenging freshwater snail family. However, it is hoped that the increasing use of molecular phylogenetic approaches will significantly enhance our understanding of ancylid systematics, ecology and invasion history.

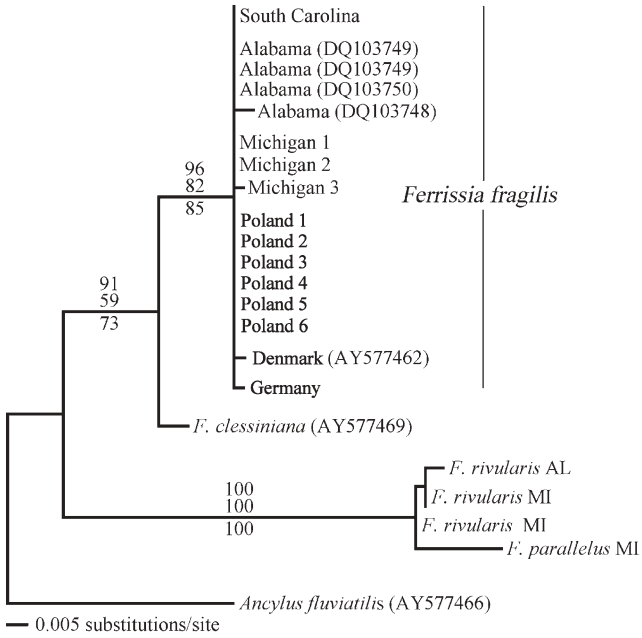

Figure 2. Bayesian phylogram of the ancylinid mitochondrial large subunit ribosomal DNA (mt 16S) dataset composed of four *Ferrissia* ingroup species and the designated outgroup *Ancylus fluviatilis*. Nodal posterior probabilities are given below, and maximum parsimony (top) and maximum likelihood (middle) bootstrap support values are presented above, the respective branches. GenBank accession numbers are given for non-novel haplotypes. All European *Ferrissia* haplotypes (sampling locations indicated in bold) nested unambiguously with the *F. fragilis* clade. The German haplotype was obtained by C. Albrecht from a specimen, identified as *F. clessiniana*, sampled in Triebes, Greiz Co., Germany.

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First report of pseudohermaphroditism in cephalopods

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Cephalopods are gonochoristic molluscs that show no hermaphroditism and the animals never change sex.[1] Their main sexual dimorphism is the presence of a hectocotylized arm in males, which transfers the spermatophores to the female. In the incirrate octopods, females have a single ovary with paired oviducts, whereas in males a single duct forms the spermatophoric complex and the terminal organ (or penis). Several malformations have been reported for cephalopods, including internal and external structures. Among others, these refer to malformations of arms,[2–6] of gills and associated vascular organs, of mantle-hyponomal locking cartilages, to dextral displacement of the caecum with respect to the location of the stomach, to duplication of the chitinous lining of the alimentary canal,[7] to the presence of double hectocotylization[6,8,9] (abnormal characteristic in incirrate octopods) and to malformation of the systemic heart complex.[10] However, so far there are no published records of pseudohermaphroditism for the class.

During a study of the reproductive biology of the Patagonian red octopus, *Enteroctopus megalocyathus* (Gould, 1852), a total of 185 females and 143 males were collected during research surveys conducted from June to December 2004 in Nuevo Gulf (42°46′ S 65°02′ W), Atlantic Ocean, Argentina, at 6 to 10 m depth. Each freshly collected animal was weighed, measured and sexed on the basis of the presence of the hectocotylus. Internal organs were removed and immediately fixed in 10% formalin, and the rest of the animal was discarded. Three months after fixation, when preparing internal organs, an abnormality was noticed in one individual.

The abnormal specimen (A) appeared in the sample of August. Because of its size and weight, and the presence of immature oocytes, it was probably immature. For this reason, we compared it with other normal immature females and males (i.e. animals with immature oocytes and without spermatophores respectively) from the same August sample (N). The following measurements and indices were recorded: total body weight, dorsal mantle length, reproductive system weight, spermatophoric complex weight, ovary weight, weight of oviducts and oviducal glands and terminal organ length. For the last four measurements, we calculated a simple 'abnormality index' as $(X_A/\bar{X}_N) * 100$, where X_A is the reproductive tissue of the abnormal individual divided by its total body weight, and $\bar{X}_N$ is the sample mean of the same reproductive tissue divided by mean total body weight for all normal individuals. X_A and $\bar{X}_N$ were used to compare statistically the reproductive tissue of the abnormal individual with that of normal ones, using a special case of the t-test.[11]

To identify the type of gonadal tissue and differences between normal specimens and the abnormal one, histological preparations of several parts of the female and male reproductive system were stained with haematoxylin and eosin. The latter system was defined according to Mann *et al.*[12]

The abnormal specimen was initially sexed as a female, i.e. it did not exhibit a hectocotylus. Internally, it showed male structures with normal genital female characteristics, orientated as in normal octopuses. In its male reproductive system, the testis was absent, the penis was normal and both the Needham sac, and glandular systems I and II displayed an abnormal shape (Fig. 1). In its female reproductive system, the ovary was significantly larger than normal (Table 1). The spermatophoric complex and left female gland and oviduct lay enclosed in a male membranous sac and were joined only between glandular system II and the left distal oviduct. The presence of immature oocytes was also evident through the ovary wall. Histological comparisons did not reveal differences from normal ovaries, and we did not find testicular tissue in the ovary nor sperm stored in oviducal glands. In view of these morphological and histological characteristics, we suggest that this individual shows the first case of pseudohermaphroditism in cephalopods.

The presence of mixed female and male structures may not have caused sterility for the female function since one oviduct was free and showed normal characteristics. Although this

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