A SYSTEMATIC STUDY OF NORTH AMERICAN FRESHWATER LIMPETS (GASTROPODA: HYGROPHILA: ANCYLIDAE)

by

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Table of Contents

List of Tables

Table

List of Figures

Figure

Abstract

The freshwater limpet family Ancylidae, comprised exclusively of patelliform taxa, has a near-cosmopolitan distribution in freshwater ecosystems. Paul Basch was the last to thoroughly study North American ancylid systematics, and I used his 1963 monograph as a guide in sampling nominal North American ancylid species and constructing representative nuclear and mitochondrial (mt) gene trees. My objectives were to a) assess the monophyly of the family Ancylidae, b) analyze intergeneric and interspecific ancylid relationships, and c) address the validity of nominal North American species. In the context of global samples, I recovered a monophyletic Ancylidae and a pronounced sub-familial dichotomy separating the New World genera (*Laevapex*, *Hebetancylus*, *Uncancylus*, *Gundlachia*) from a Holarctic sister clade (*Ferrissia*, *Rhodacmea*, *Ancylus*). While support for the two ancylid subfamilies was robust, support for specific intergeneric relationships within the subfamilies was lacking. In my gene trees, all nominal *Laevapex* species emerged as a single lineage, suggesting that the North American *Laevapex* is monotypic, comprised of only *L. fuscus*. This finding was corroborated by a geometric morphometric analysis of shell vouchers that indicated no difference in shell shape among the nominal species. Rare, highly divergent mt lineages in some *Laevapex* populations likely originated from either introgression or from persistent ancestral polymorphisms. Only two of 36 *L. fuscus* mt haplotypes occurred in multiple populations, indicating that long distance dispersal is rare, and field observations of phoresy on the giant water bug *Belostoma flumineum* identified it as a potential agent

of local dispersal. North American *Ferrissia* samples formed two major clades, corresponding to the type species *F. rivularis* and the widespread *F. fragilis*. The former exhibited pronounced east-west geographic structure, with evidence of secondary transcontinental movement from the east to the west but no indication of introgression; thus, these lineages may be speciating. North American *F. fragilis* has cryptically invaded Europe and Asia, providing striking evidence of human-mediated intercontinental dispersal in ancylids, a feature that has further complicated systematic studies of this group.

Chapter I

The family Ancylidae

Introduction

The freshwater limpet family Ancylidae is among the most ubiquitous of all freshwater gastropods, with representatives on every continent except Antarctica (Basch, 1959, 1963; Burch, 1982) and occurring in a variety of habitats ranging from permanent ditches to fast-flowing streams to the still edges of large rivers and lakes (Basch, 1963; Burch, 1982). Morphologically, ancylids are distinguishable from most other snails because they all possess cap-shaped or patelliform shells (Basch, 1963; Brandt, 1974; Hubendick, 1979; Burch, 1982, 1988), generally with low profiles and enlarged apertures, as opposed to the coiled shells of most gastropods; this morphotype has earned them the common name "limpet." As pulmonates, or snails that have developed a mantle cavity lung, they have thin, light shells, lack an operculum, and are hermaphroditic (Perez et al., 2004).

Ancylids are sinistral, or left-handed, in body organization (Burch, 1982; Burch and Jung, 1992), thus all major body openings into the mantle cavity (e.g., pulmonary, renal, alimentary, and reproductive) are located on the left side of the animal (Basch, 1959; Burch, 1982; Burch and Jung, 1992), and their shell apex is located right of the midline when viewed from above (Burch, 1982). To supplement the reduced mantle cavity lung, they have a secondarily-derived pseudobranch or false gill for gas exchange (Pilsbry and

Bequaert, 1927; Basch, 1963; McMahon, 1975; Burch, 1982), a structure also found on the left side of the body (Burch, 1982). This character they share with members of the Planorbidae, a closely related (Hubendick, 1979; Burch, 1982) higher limnic basommatophoran family, and the Acroloxidae, a more primitive basommatophoran lineage comprised exclusively of patelliform snails (Burch, 1988).

It is important to note that the limpet morphotype is not restricted to the family Ancylidae. Several highly divergent freshwater and marine gastropod taxa exhibit patelliform shells (Basch, 1963), and at least among the freshwater limpets, convergent evolution is believed to be the driving force (Albrecht et al., 2004, 2007). Having this shell morphology is advantageous because the entire soft body of the animal is enveloped when it is firmly attached to a substrate, thus staving off threats such as desiccation, predation, and dislodgement in lotic environments (Burch, 1988; Burch and Jung, 1992; Albrecht et al., 2004). In North America, two other freshwater gastropod families have extant limpet representatives, including the Lymnaeidae and the aforementioned Acroloxidae (Burch, 1982). Patelliform members of these two taxa are unambiguously distinct from ancylids primarily because they have a dextral, or "right-handed," body organization with all body orifices on the right (Basch, 1963; Burch, 1982, 1988; Paul and Clifford, 1991), and their apex is just left of the midline (Burch and Tottenham, 1980). The Planorbidae once possessed an extant limpet-form taxon, *Amphigyra alabamensis*, but that species is now extinct due to pollution in the southeast United States where it was found (Burch, 1988).

Ancylids play a fundamental role in their ecosystems both as prey for larger invertebrates, fish, and waterfowl, and as grazers that control algal growth. Ancylids are also considered of medical importance because they are capable of serving as temporary hosts for trematode parasites (Basch, 1963; Morgan et al., 2002; Thiengo et al., 2004). Despite their ecological and medical significance, however, this family has received relatively little attention in the freshwater gastropod literature in recent decades, and persistent systematic uncertainties have only complicated the few studies that have been attempted (Jørgensen et al., 2004; McMahon, 2004). Unfortunately, the pollution and damming of North American watersheds due to 20th century industrialization has led to the extinction of many species of freshwater mollusks (Bogan et al., 1995; Lydeard and Mayden, 1995; Burkhead et al., 1997; Master et al., 2000; Lydeard et al., 2004), and ancylids are among the groups impacted; thus, the need for a taxonomic revision of this group is more pressing than ever if attempts at preserving its diversity are going to be made.

Ancylids of North America

The first ancylid discovered in the Americas (in Indiana) was *Ancylus rivularis* (Say, 1817), now known as *Ferrissia rivularis*. This event prompted the discovery of more limpets throughout the United States over the next hundred years. Pilsbry and Walker are responsible for naming and describing many North American ancylid species in the early 1900s (Walker, 1918; Basch, 1963; Thompson, 1999), and Basch (1963) was the last to exhibit significant interest in the taxonomy of the group. In a taxonomic revision completed by Basch, he relies strictly upon morphological variation (e.g. differences in

size, pigmentation, shell contour and sculpture, adductor muscle arrangement, pseudobranch morphology, radular characteristics, male reproductive anatomy, etc.) to classify North American ancylids. His monograph is now quite dated considering that more reliable molecular approaches for resolving systematic issues have developed since his time, though his study serves as an invaluable guide for addressing the lingering systematic uncertainties afflicting the group.

Basch (1963) recognized four genera of North American ancylids (e.g., *Laevapex* Walker 1903, *Ferrissia* Walker 1903, *Rhodacmea* Walker 1917, and *Hebetancylus* Pilsbry 1913), the same four that are widely recognized today (Burch, 1982, 1988; Burch and Tottenham, 1980). *Laevapex* is widespread throughout the eastern United States, occurring in lakes and slow-flowing rivers (Basch, 1963; McMahon, 1975; McMahon and Aldridge, 1976; Burch, 1982), and members of the genus are characterized by their smooth apex (Burch, 1982; Thompson, 1999). Generally, two species of *Laevapex* are recognized, including *L. fuscus* and *L. diaphanus* (Basch, 1963; Burch 1982; Burch and Tottenham, 1980), though Thompson recognizes three, with the third being *L. peninsulae* (Thompson, 1999).

The most widespread and diverse genus, *Ferrissia*, has a near-cosmopolitan distribution, occupying streams and standing water around the world (Basch, 1963; Burch, 1982). More locally, they may be found throughout the United States and extend into Canada and Mexico. Members of the genus are recognized by fine radial striate on the shell apex (Basch, 1963; Burch, 1982; Thompson, 1999). Because of their minute size and ability to

survive outside of water when attached firmly to a hard surface, *Ferrissia* are easily passively transported (Burch, 1982). Five *Ferrissia* species are commonly recognized, including *F. rivularis*, *F. fragilis*, *F. parallela*, *F. walkeri*, and *F. mcneilli* (Basch, 1963; Burch, 1982; Burch and Tottenham, 1980), though motions to pull others out of synonymy have been made (Thompson, 1999)

Rhodacmea is a genus restricted to lotic habitats in the southeastern United States (Basch, 1963; Burch, 1982). Constituents are known for having elevated, radially striate shells with a slight pink color at the shell apex, which is frequently worn in older specimens (Burch, 1982). Three species are recognized, including *R. filosa*, *R. hinkleyi*, *R. elatior* (Basch, 1963; Burch, 1982; Burch and Tottenham, 1980) though members of the genus are extremely elusive, and some may now be extinct due to the extensive damming of rivers in the south (Basch, 1963). *Rhodacmea* is believed to exhibit a primitive limpet morphology, while the other North American genera are considered more derived (Burch, 1988). It is likely closely related to the Eurasian and North African ancylid *Ancylus* based on similarities observed in the soft anatomy and shell sculpture (Burch, 1962, 1974, 1982).

Finally, *Hebetancylus* is a tropical genus with a restricted range in the southeast United States (Burch, 1982) that extends down into Mexico and Central America (Basch, 1963; Hubendick, 1967; McMahon and Aldridge, 1976; Burch and Tottenham, 1980). Much like *Laevapex*, members of the genus have a smooth apex, though in *Hebetancylus* it is far right of center (Burch, 1982; Thompson, 1999). One species in generally recognized

in North America: *Hebtancylus excentricus* (Basch, 1963; Burch, 1982; Burch and Tottenham, 1980).

Purpose of this study

Intrigued by the widespread, yet elusive nature of freshwater limpets, I have chosen to address some of the long-standing uncertainties and inconsistencies regarding North American ancylid systematics. Moreover, the initiation of effective conservation efforts requires that such topics be considered. Namely, my study aims to (1) determine the phylogenetic positioning of Ancylidae in relation to the other higher limnic Basommatophora, (2) identify which nominal ancylid genera and species are phylogenetically valid, focusing largely on North American taxa, and (3) determine how these ancylid genera and species are interrelated from an evolutionary viewpoint. To address these issues, I have acquired samples of as many nominal North American ancylid species as possible, from as broad a range as possible, primarily using Basch's system of classification as a guide. I use innovative molecular techniques (e.g. DNA amplification and sequencing) to uncover genetic variation and infer evolutionary relationships among ancylids, an approach that alleviates much of the fallibility intrinsic in using nondiscrete, oftentimes environmentally-induced morphological variation. Of course, morphological variation should never be completely disregarded, and after completing my molecular phylogenetic studies, I relate my findings back to the original morphological observations.

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Chapter II

E Pluribus Unum: A Phylogenetic and Phylogeographic Reassessment of *Laevapex* (Pulmonata: Ancylidae), a North American Genus of Freshwater Limpets

Abstract

The North American freshwater limpet genus *Laevapex* (Walker, 1903) is a ubiquitous inhabitant of lentic and slow-moving lotic habitats east of the Rocky Mountains, but uncertainty clouds its systematic affinities, the phylogenetic validity of its constituent nominal species, and its degree of genetic connectivity among drainages. We addressed these issues by sampling the genus throughout much of its collective range and constructing representative nuclear and mitochondrial (mt) gene trees, in addition to performing morphometric analyses of shell shape variation. Our results identify neotropical *Gundlachia* and South American *Uncancylus* as sister lineages for *Laevapex* and reveal a pronounced sub-familial dichotomy within the Ancylidae, separating these three New World genera from a Holarctic (*Ferrissia* (*Ancylus*, *Rhodacmea*)) sister clade. Five nominal taxa (*L. fuscus*, *L. diaphanus*, *L. peninsulae*, *L.* sp., and "*F.*" *arkansasensis*), indistinguishable in our morphometric analyses, were polyphyletic in the mt gene trees, exhibited modest levels (<3.9%) of genetic divergence in the primary (103 of 109 individuals) mt clade and, with one minor exception, they appeared fixed for a single nuclear ITS-2 genotype. Although complicated by the presence of rare, highly divergent mt lineages (of either introgressive or persistent ancestral polymorphic origin) in some populations, the molecular data were consistent with a taxonomic conclusion that

these five nominal taxa represent a single polymorphic lineage of the type species *L. fuscus*. AMOVA analyses indicated that 56% of the observed mt variation could be attributed to among population differences, only two of 36 haplotypes were detected in more than one sampling location, and estimates of among-population mt gene flow were generally low at both regional and continental scales. Unrooted network analyses revealed a number of mt tip clades, one restricted to the southwestern part of the range, the remainder having overlapping distributions in eastern North America. All of the eastern tip clades occurred in the mid-Atlantic region, and these samples displayed by far the highest levels of collective mt diversity. However, directional gene flow estimates indicated that this region has been a recipient (especially from Alabama populations), rather than a source of haplotypic diversity, implying that it likely represents a center of overlap, not a primary ice age refugium, for this limpet species.

Introduction

During the last century, the freshwater malacofauna of North America experienced a major wave of extinction as a consequence of extensive watershed industrialization (Bogan et al., 1995; Lydeard and Mayden, 1995; Burkhead et al., 1997; Master et al., 2000; Lydeard et al., 2004) — only 2% (<100,000 km) of high-quality, free-flowing rivers remain of an estimated original U.S. figure of 5,200,000 km (Benke, 1990). Fortunately, in recent years, there has been a renaissance in the scientific and conservation biology study of this heavily impacted fauna. This is exemplified by the establishment in 1998 of the *Freshwater Mollusk Conservation Society* and by the resurgence of significant research activity on major taxa such as unionid mussels

(Lydeard and Roe, 1998; Graf and Ó Foighil, 2000; Roe et al., 2001; Kandl et al., 2001; Buhay et al., 2002; Serb et al., 2003; Beaty and Neves, 2004), pleurocerid gastropods (Lydeard et al., 1997; Holtznagel and Lydeard, 2000; Minton, 2002; Minton and Lydeard, 2003), and sphaeriid clams (Lee and Ó Foighil, 2002, 2003; Guralnick, 2004; Lee, 2004).

However, this renewed interest has not yet been extended to all ecologically prominent groups of North American freshwater mollusks, least of all to freshwater limpets, a heterogeneous basommatophoran assemblage that last received serious attention over 40 years ago (Basch, 1959, 1960, 1962a, 1962b, 1963a, 1963b). Taxonomic uncertainty pervades the North American freshwater limpet literature, and it is quite common for study taxa to be identified to subfamilial (McMahon, 2004) or to generic (Morgan et al., 2002) status only. This unsatisfactory situation stems in large part from inadequate generic and species-level descriptions (Basch, 1963b). Other potential contributing factors include the pronounced ecophenotypic plasticity in shell morphology common to many freshwater limpet species (Basch, 1963a, 1963b; Durrant, 1975; Sutcliff and Durrant, 1977; McMahon and Whitehead, 1987; McMahon, 2004), together with the possibility of latent cryptic species complexes (Pfenninger et al., 2003).

A comprehensive reassessment of the North American patelliform Basommatophora is beyond the scope of this present study, and we have focused our attention on *Laevapex* (Walker, 1903), an ancylid genus commonly found east of the Rocky Mountains in lentic and slow-moving lotic habitats such as ponds, lakes, and languid rivers (Walker, 1903;

Basch, 1963b; McMahon, 1975; McMahon and Aldridge, 1976; Burch and Jung, 1992). *Laevapex* species have fully aquatic respiration, lack the ancestral mantle-cavity pulmonate lung, and possess a distinctive secondary bi-lobed "pseudobranch" gill composed of a small dorsal lobe and a much larger ventral respiratory lobe (Basch, 1959). Based primarily on this character, Basch (1963b) proposed that *Laevapex* is sister to the tropical American genus *Hebetancylus* rather than to either of the co-occurring ancylid genera *Rhodacmea* and *Ferrissia*. Two recent molecular studies have incorporated *Laevapex* genotypes, and although both support its placement in the Ancylidae, neither identify a convincing sister lineage for this genus (Morgan et al., 2002; Albrecht et al., 2004).

There is little consensus in the limited North American ancylid literature concerning the number of *Laevapex* species, their diagnostic morphological differences (see Materials and Methods for details), and their respective distributions. Basch (1963b) recognized two species: the type species *L. fuscus* and *L. diaphanus*, both relatively widespread in eastern North America. He synonymized *L. peninsulae* with *L. fuscus*; however, the former nominal species has been re-established by Thompson (1999) and is restricted to the Floridian peninsula south and east of the Suwannee River system. This study aimed to revisit *Laevapex* systematic and phylogeographic relationships using morphometric analyses and molecular phylogenetic datasets generated for nuclear and mitochondrial (mt) markers. Issues addressed included the placement of the genus *Laevapex* within the Ancylidae, the phylogenetic validity of nominal *Laevapex* taxa, and the phylogeographic relationships of populations sampled throughout the generic range.

Materials and Methods

Specimen collection

Table 2.1 provides sampling and voucher information for all specimens sequenced in this study. Upon collection, all specimens were preserved in 95% ethanol. Samples were identified to genus based on the following *Laevapex* characters: shell apex just to the right of the midline; shell surface displaying prominent concentric growth lines, sometimes with radial sculpture, but lacking apical radial microsculpture; three adductor muscles interconnected by adhesive epithelium running between; a core of dark pigment sometimes present in the tentacles; a bi-lobed pseudobranch (Basch, 1963b; Burch, 1988).

Laevapex specimens were subsequently given a nominal species designation based on original species descriptions, relying heavily upon shell shape (as viewed from above) and on radial sculpture (Table 2.2). Figure 2.1 shows collection localities for *Laevapex fuscus, L. diaphanus*, *L. peninsulae*, *Laevapex* from Oklahoma (=*Laevapex* sp.), and "*Ferrissia*" *arkansasensis*. For the latter samples, identification to genus and species was seriously complicated by suboptimal species descriptions and taxonomic inconsistencies in the literature. For instance, Basch (1963b) synonymized "with hesitation" *Ferrissia arkansasensis* with *F. rivularis*, without examining its anatomy and based primarily on Walker's conchological description and generic placement. However, examination of the *F. arkansasensis* holotype (UMMZ 100320) suggested that it is actually a misclassified *Laevapex* (Burch, unpubl. observ.). In addition to possessing *Laevapex* shell features, individual specimens in our sample of *F. arkansasensis* topotypes from the White River

had a bi-lobed pseudobranch (Walther and Burch, unpubl. observ.), a characteristic of *Laevapex* but not of *F. rivularis* (Basch, 1963b). In this paper, we refer to the White and Cossatot River samples as "*Ferrissia*" *arkansasensis*. Additionally, limpets sharing this morphology in nearby Texan and Oklahoman populations have previously been identified as *Hebetancylus excentricus* (Morelet, 1851) (McMahon and Aldridge, 1976; McMahon, 2002), but more recently (McMahon, 2004), they have been left unassigned within the subfamily Laevapecinae. A close look at McMahon's samples from Oklahoma revealed that they also are *Laevapex* (Walther and Burch, unpubl. observ.), but no definitive species designation was applied considering that available *Laevapex* species descriptions seemingly rendered identification arbitrary. Therefore, the Oklahoma specimens were referred to as *Laevapex* sp. in this paper.

Geometric morphometrics

We analyzed shell apertural variation among the nominal species of *Laevapex* by performing morphometric analyses of 69 genotyped individuals: 12 *Laevapex fuscus*, 18 *L. diaphanus*, 4 *L. peninsulae*, 9 *Laevapex* sp., and 26 "*Ferrissia*" *arkansasensis*. The holotype specimens of *L. fuscus* and "*F.*" *arkansasensis*, and 5 supplementary non-genotyped *L. fuscus* specimens, were additionally incorporated into the analysis. Shell shape was digitized by scanning shells (Epson Perfection 1200U), aperture side down, and then tracing the scanned images using ImageJ 1.34g (Abramoff et al., 2004). A "fan" of one hundred radiating lines was added to all 76 shell images using Makefan 6 (available at http://www2.canisius.edu/~sheets/morphsoft.html). Radiating lines were of equal angular intervals, and the points where they met the margin of the shell constituted

semilandmarks (Bookstein, 1997; Zelditch et al., 2004) that defined the curvature of the shell outline. Semilandmarks were digitized using tpsDig 1.40 (available at http://life.bio.sunysb.edu/morph/) for a total of 50 semilandmarks per shell.

Because semilandmarks were used in this analysis, the data also had to be adjusted to account for the arbitrary spacing of the semilandmarks along the curve of the shell. The methodology used was to slide semilandmarks to a perpendicular alignment along a reference curve, a method that uses the minimum distance among shapes as its criterion for an optimal superimposition (SemiLand 6, available at http://www2.canisius.edu/~sheets/morphsoft.html; Bookstein, 1997; Zelditch et al., 2004). The reference curve was obtained from the collective dataset. Generalized least squares (GLS) procrustes superimposition (CoordGen 6, available at http://www2.canisius.edu/~sheets/morphsoft.html) was used to remove nonshape variation (*e.g.*, position, orientation, and size) from the shape data. Because this procedure produces coordinates with four too many degrees of freedom, the coordinates obtained from GLS were transformed into partial warp scores and scores on the uniform component of shape. Partial warp scores are components of the thin-plate spline interpolation function describing the non-affine part of shape variation; the affine part is described by the uniform component (Bookstein, 1991; Zelditch et al., 2004). Shape variation among shells was analyzed using a principal components analysis (PCA) (PCAGen 6, available at http://www2.canisius.edu/~sheets/morphsoft.html).

DNA sequencing

Total genomic DNA was extracted from specimens using a DNeasy Tissue Kit (Qiagen). In most cases the entire body of the animal, minus the shell, was used for processing. For larger specimens, only a small sample of tissue from the foot was used. Three markers were selected for sequencing in this study: nuclear ribosomal large-subunit (28S), mt *cytochrome oxidase I* (COI), and nuclear second internal transcribed spacer (ITS-2). Table 1 indicates which samples were sequenced for each gene, including how many individuals from a sample were sequenced and the number of unique genotypes obtained per sampling locality. To amplify the target fragments, the polymerase chain reaction (PCR) was applied using Go*Taq* polymerase (Promega) and the following primer pairs: D23F D6R (Park and Ó Foighil, 2000) for 28S; LCO1490 HCO2198 (Folmer et al., 1994) for COI; and ITS-2F ITS-2R (Xu et al., 2001) for ITS-2. LCO1490 and HCO2198 failed to amplify mt COI from many of our samples, leading us to design a supplementary *Laevapex*-specific COI primer pair, internal to the Folmer et al. (1994) primers: LCO-LAEV (5'-atattggacaytatatttag-3') and HCO-LAEV (5'-aaatcaaaataartgttgat-3'). A touchdown protocol (Palumbi, 1996) was used for all amplifications, each run including a negative control (lacking template) to check for contamination. For 28S and ITS-2, PCR conditions included an initial annealing temperature of 65°C that was decreased by 2°C/cycle until the final annealing temperature (48-50°C) was reached and maintained for an additional 30 cycles. For COI, an initial annealing temperature of 53°C was decreased by 1°C/cycle until the final annealing temperature (43-45°C) was reached and maintained for an additional 30 cycles. After being isolated by gel electrophoresis on a 1% agarose gel, PCR products were

excised over UV light and cleaned using either a QIAEX II Gel Extraction Kit (Qiagen) or a QIAquick Gel Extraction Kit (Qiagen) and directly sequenced at the University of Michigan Sequencing Core.

Phylogenetic analyses

The resulting sequence chromatograms were revised manually in Sequence Navigator 1.0.1 (Applied Biosystems) by comparing the forward and reverse strands for each specimen. The edited sequences were then aligned for each gene separately using ClustalW (Thompson et al., 1994) implemented in Sequence Navigator and, where needed, the alignment was modified by hand. Our sequences were deposited in GenBank (DQ328274-328299 for 28S, DQ328221-328273 for COI, and DQ328300-328316 for ITS-2). For 28S and COI, supplementary sequences were downloaded from GenBank and incorporated into the aligned datasets. The final aligned data matrices were 806 bp in length for 28S, 670 bp for COI, and 661 bp for ITS-2. Genotypes for each of the three datasets were assembled in a NEXUS format using Sequence Monkey 2.9.0 (available at http://sequence_monkey.tripod.com/). Phylogenetic analyses were carried out using PAUP* 4.0b10 (Swofford, 2003). The maximum parsimony (MP) analysis for each of the three datasets was conducted as a heuristic search using equal character weighting, 100 random stepwise addition and tree-bisection-reconnection (TBR) branch-swapping. Inferred sequence gaps were coded as missing data. Bootstrap (Felsenstein, 1985) branch support levels were estimated with 1000 replications, 10 random additions each. For maximum likelihood (ML) analyses, the best-fit model for each of the three datasets (GTR+G+I for 28S, GTR+G+I for COI, and K80(K2P)+G for ITS-2) was obtained in

Modeltest 3.06 (Posada and Crandall, 1998). In each case, one of the MP trees was used as a starting tree with the TBR branch-swapping algorithm in PAUP*. Bootstrap support values were generated using a fast-heuristic search with 100 replicates. In MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003), Bayesian searches for each dataset were run for $1x10^6$ generations using the same best-fit models as in the ML analyses. Posterior probabilities were calculated by creating a majority-rule consensus for every 100^{th} tree after burn-in of the first 2,000 trees using PAUP*.

Genetic Structure and Migration Rates

Laevapex crown clade mt COI genotypes (excluding the 4 divergent haplotypes) were combined into five regional groupings: 1) Arkansas + Oklahoma (AR/OK); 2) Alabama; 3) South Carolina + Virginia (SC/VA); 4) Michigan + Minnesota (MI/MN); and 5) Florida, and their respective genetic structures were characterized using Arlequin 2.001 (Schneider et al., 2000). Genetic variation was estimated using haplotype diversity (*H*; Nei, 1987) and nucleotide diversity (π, the mean of pairwise sequence differences; Tajima, 1983). The fraction of the total genetic variation distributed among populations was estimated with the analysis of molecular variation (AMOVA; Excoffier et al., 1992) test based on simple pairwise distance.

We estimated patterns of gene flow among the regional groups using Migrate 1.7.6 (Beerli and Felsenstein, 2001), which calculates directional and asymmetric gene flow using a Markov chain Monte Carlo maximum-likelihood procedure. A maximum-likelihood (ML) corrected transition to transversion ratio, calculated from the Bayesian

tree using PAUP*, and empirical base frequencies were used. Each search run involved 10 short chains with 100,000 sampled genealogies and 3 long chains with 1,000,000 sampled genealogies. For both the short and long chains, 10,000 genealogies were discarded as initial burn-in. A static heating scheme, four chains with temperatures 1.0, 1.2, 1.5, and 3.0, was used. Three initial searches were conducted for each clade with different random seeds and F_{st}-based estimates of Θ (= $2N\mu$ for the mtDNA genome, where N = the effective population size and μ = the mutation rate) and migration rates ($M = 2mN$) in order to check consistency of the results. Three additional runs were performed with parameter estimates from the previous run as starting values.

Results

Geometric morphometrics

Variation in the ratio of shell length to width (principal component 1 or PC1) constituted almost all (83.7%) of the variance in apertural shape. Shell apertures with high scores for PC1 were elongate, while apertures with low PC1 scores were nearly circular. In Fig. 2.2, PC1 values are plotted against the next highest principal component (PC2; 3.5% of total variance) that described minor variation in the anterio-lateral concavity and posterior convexity of the shell profiles. As is evident from Fig. 2.2, the nominal *Laevapex* taxa did not form exclusive clusters along PC1, implying that apertural shape, the primary conchological feature used to discriminate the nominal taxa (Table 2.2), fails to separate them.

Phylogenetic relationships of the Ancylidae - 28S rDNA

Figure 2.3 shows the Bayesian tree obtained for our 28S nuclear rDNA dataset comprised of 13 *de novo* and 14 GenBank genotypes. MP and ML analyses resulted in similar topologies (not shown), differing in relatively minor details as outlined below. The marine pulmonate *Siphonaria pectinata* (Linnaeus, 1758) was employed as an outgroup (Dayrat et al., 2001; Remigio and Hebert, 2003), and the ingroup taxa contained representatives of six freshwater basommatophoran families: Chilinidae, Lymnaeidae, Physidae, Acroloxidae, Planorbidae, and Ancylidae. A number of well-supported internal nodes distinguished some of the ingroup taxa, including *Chilina*, which occupied a basal position. The most robust stem node separated the Planorbidae and Ancylidae from representatives of the Acroloxidae, Physidae, and Lymnaeidae. The latter two families formed a convincing tip sister pair for all analyses although the acroloxid clade varied in its placement: weakly sister to Physidae and Lymnaeidae in the MP tree or basal to them in the Bayesian (Fig. 2.3) and ML topologies.

In all of our analyses, the 28S dataset recovered a well-supported planorbid/ancylid clade containing a robustly monophyletic Ancylidae. However, details of the topological inter-relationships between these two families varied according to analytical method. A basal ancylid/planorbid polytomy was recovered in Bayesian analyses (Fig. 2.3), whereas in both MP and ML analyses (not shown), the ancylid clade was tenuously (bootstrap [BS] support values <50 in the MP tree) nested within the planorbid clade, sister to *Amerianna*, *Planorbula*, and *Biomphalaria*. Within the ancylid clade, the 28S data yielded congruent topologies for all three analytical methods. A robust dichotomy

distinguished *Laevapex*, *Gundlachia*, and *Uncancylus* from a sister clade comprised of a paraphyletic *Ferrissia* and a tip clustering of *Rhodacmea* and *Ancylus* (Fig. 2.3). Interestingly, the South American *Uncancylus* sp. possessed a bi-lobed pseudobranch similar in morphology to that of *Laevapex* and *Gundlachia* (Hubendick, 1964), an apparent synapomorphy not present in the other ancylid subclade (Basch, 1959). All nominal *Laevapex* taxa, in addition to Cossatot River "*Ferrissia*" *arkansasensis*, shared an identical 28S rDNA genotype, although minor variants, differing by 1-3 substitutions, were also present.

Phylogenetic status of nominal *Laevapex* species – mt COI and nuclear ITS-2

The 28S rDNA data (Fig. 2.3) yielded valuable insights into deeper ancylid cladogenic relationships but were relatively uninformative concerning those of the nominal *Laevapex* species. To generate more generic level resolution for the focal study taxa, we characterized a cumulative total of 109 nominal *Laevapex fuscus* (N=28), *L. diaphanus* (N=28), *L. peninsulae* (N=7), *Laevapex* sp. (N=7), and "*Ferrissia*" *arkansasensis* (N=39) individuals, sampled from 14 regional sampling locations (Fig. 2.1; Table 2.1), for the target mt COI fragment, yielding 36 unique haplotypes. Novel homologous sequences were also generated for other freshwater limpets (*Uncancylus* sp., *F. fragilis*, *F. rivularis*, *F. parallela*, *Ancylus fluviatilis*, *Rhodacmea elatior*, *Acroloxus lacustris*, and *A. coloradensis*) and supplemented with 8 GenBank basommatophoran COI genotypes. Phylogenetic analyses of the COI dataset generated gene trees that were broadly congruent with the 28S trees (Fig. 2.3) for major nodes of interest: a monophyletic Ancylidae nested within the Planorbidae; a stem ancylid dichotomy distinguishing

Ferrissia/Ancylus/Rhodacmea from *Laevapex/Uncancylus*; placement of "*F.*" *arkansasensis* within the *Laevapex* clade (Fig. 2.4a).

The robust (MP BS=95) crown *Laevapex* mt COI clade (Fig. 2.4a) exhibited three striking characteristics. Firstly, none of the constituent nominal taxa (*Laevapex fuscus*, *L. diaphanus*, *L. peninsulae*, *Laevapex* sp. and "*Ferrissia*" *arkansasensis*) were monophyletic. Secondly, although the large majority of haplotypes formed a shallow tip clade characterized by short internal branches, a small minority (N=4) of rare divergent haplotypes formed a topologically distinct sister clade (in ML and Bayesian analyses only, were paraphyletic in MP strict consensus trees) containing much longer internal branches. These divergent haplotypes were found in low frequencies distributed among 4 nominal taxa. Thirdly, a minority of crown clade COI genotypes (N=4) exhibited a small number of inferred amino acid deletions clustered in a 7 amino acid segment of the protein (Fig. 2.5), a feature not observed in the remainder of the ancylid COI dataset (Fig. 2.4a). These inferred deletions did not interrupt the COI reading frame, and although three of the four occurred in divergent clade haplotypes, the fourth involved a shallow tip clade haplotype (Fig. 2.4a). The latter case is particularly interesting because it involved a single inferred amino acid (alanine) deletion that showed population-level variation, in the absence of apparent nucleotide substitutions, in the Cossatot River (Arkansas) sample of "*F.*" *arkansasensis* (Fig. 2.5). Four individual limpets exhibited this deletion, but they otherwise showed no difference from the most common (11/28 limpets) COI haplotype in this population (Table 2.1; Figs. 2.4a, 2.5).

We investigated whether the low frequency presence of divergent mt COI genotypes in the study populations (Table 2.1; Fig. 2.4a) corresponded with within-population nuclear genome diversity by cross-referencing the mitochondrial dataset with a nuclear marker. A sub-sample of 17 individuals, chosen to represent the primary *Laevapex* COI clade topological features (including 4 specimens representing the 4 divergent haplotypes) and the main sampling locations, had their nuclear ribosomal second internal transcribed spacer (ITS-2) region sequenced. All typed limpets shared a single ITS-2 genotype with the exception of one *L. peninsulae* individual sampled in Collier County, Florida, that differed by 4 nucleotides (Fig. 2.4b). Thus, the mitochondrial and nuclear data indicate that all nominal *Laevapex* species represent a single polymorphic lineage and are hereby collectively synonymized with *L. fuscus*.

Laevapex phylogeography

A ML analysis of the *Laevapex* mt COI crown clade, minus the 4 divergent haplotypes (Fig. 2.4a), is shown as an unrooted network in Fig. 2.6. Note that, as in the rooted tree (Fig. 2.4a), none of the nominal taxonomic designations formed exclusive clades and also that all but 2 of the 36 haplotypes recovered were restricted to single sampling locations. There was little phylogeographic structure evident apart from the Arkansas and Oklahoma samples that formed a shallow southwestern tip clade (if the two divergent haplotypes present in these populations [Fig. 2.4a] are ignored). The other tip clades exhibited overlapping distributions in eastern North America; indeed all were collectively encountered in the mid-Atlantic (Virginia + South Carolina) samples. The eastern tip clades were geographically widespread, the most extensive being the nominal *L. fuscus/L.*

diaphanus tip clade that was recovered from the Minnesota, Michigan, Virginia (New River), South Carolina (Sugar Creek), and Alabama (Cahaba River) samples (Fig. 2.4a), spanning all major eastern North American watersheds. The longest and most robust (BS=100) internal branch separated one of the eastern tip clades, composed of haplotypes from three nominal species sampled from South Carolina (*L. diaphanus*, Sugar Creek), Virginia (*L. fuscus*, South Anna River) and Florida (*L. peninsulae*), from the remainder of the network.

To estimate directional gene flow for *Laevapex* across its eastern North American range, the crown clade COI genotypes were first combined into five regional groupings in order to consolidate sample sizes: 1) Arkansas + Oklahoma (AR/OK); 2) Alabama; 3) Mid-Atlantic (SC/VA); 4) Michigan + Minnesota (MI/MN); and 5) Florida (Table 2.3). Note that, as was also evident in the network (Fig. 2.6), the SC/VA combined sample was by far the most heterogeneous (π=11.248). AMOVA analyses indicated that 56% of the observed variation could be attributed to among population differences, and estimates of directional gene flow were generally low, with the exception of a high rate (3.50) from Alabama to SC/VA (Table 2.4). The Florida sample was the least diverse, being fixed for 1 haplotype (Table 2.3), and the most isolated, receiving an estimated migration rate of 0.03 from SC/VA (Table 2.4). The western AR/OK grouping was the next most isolated, its greatest connection being the recipient of 0.15 from MI/MN. There was negligible east-west gene flow between our two most densely sampled regional locations: AR/OK and SC/VA. In order to estimate within-region gene flow, we also calculated the connectivity among our three individual sampling locations in AR/OK and three others in

SC/VA (Table 2.4). Inferred rates were again predominantly low, with the exception of a heightened value (1.24) detected from the Oklahoma site to the Cossatot River in Arkansas.

Discussion

Ancylid relationships

Our nuclear and mt gene trees corroborate basommatophoran molecular phylogenetic studies that nest Ancylidae within the planorbid clade (Morgan et al., 2002; Remigio and Hebert, 2003; Albrecht et al., 2004) and extend the initial (*Laevapex* (*Ancylus*, *Ferrissia*)) phylogeny of Albrecht et al. (2004) to reveal a pronounced dichotomy separating the New World *Laevapex*, *Gundlachia*, and *Uncancylus* from a Holarctic (*Ferrissia* (*Ancylus*, *Rhodacmea*)) sister clade (Figs. 2.3, 2.4a). Although we were unable to obtain verified specimens of *Hebetancylus excentricus* (hypothesized as the sister lineage of *Laevapex* by Basch [1963b]) for this study, our result identifying the neotropical/South American ancylid genera *Gundlachia* and *Uncancylus* as *Laevapex* sister lineages is consistent with Basch's (1963b) hypothesis that *Laevapex* had a neotropical origin. Future research will likely reveal that this New World *Laevapex*/*Gundlachia Uncancylus* ancylid clade, which we refer to here as the subfamily Laevapecinae, is widespread in the neotropics and may lead to the phylogenetic and taxonomic incorporation of additional nominal neotropical ancylid genera such as *Hebetancylus* and *Anisancylus*. Based on the available data, it seems that a prominent bi-lobed pseudobranch, known from *Laevapex* (Basch, 1959; Hubendick, 1964), *Uncancylus* (Marcus and Marcus, 1962; Hubendick, 1964; this study), *Hebetancylus* (Basch, 1963b; Hubendick, 1964), *Gundlachia*

(Hubendick, 1964), and *Anisancylus* (Hubendick, 1964), may represent a laevapecinid synapomorphy.

There has been considerable speculation concerning the systematic relationships of *Rhodacmea*, an endangered endemic genus of southeastern North American limpets, which contains six currently recognized species that are restricted to fast flowing streams and rivers (Basch, 1962b, 1963b). Zilch (1959), emphasizing shell characteristics, raised *Rhodacmea* to family status (Rhodacmeidae) and placed it at the base of the patelliform ancylids. A sister status for *Rhodacmea* and the Old World *Ancylus* was proposed by Burch et al. (1960) and by Basch (1963b), based on conchological and radular characteristics, and this linkage has additionally been supported by karyological (Burch et al., 1960; Burch, 1965; Patterson and Burch, 1978) and apical shell sculptural (Burch, 1974) similarities. Hubendick (1979) emphasized the importance of a number of *Rhodacmea* anatomical distinctions and placed it in its own subfamily, sister to the remainder of his Ancyloplanorbidae. Our phylogenetic trees (Figs. 2.3, 2.4a) are the first to incorporate *Rhodacmea* genotypes, and they unambiguously support an *Ancylus/Rhodacmea* sister relationship (Burch et al., 1960; Basch, 1963b; Burch, 1965, 1974; Patterson and Burch, 1978) as part of an ancylid Holarctic clade also containing *Ferrissia* species. *Ancylus fluviatilis* is the type species of the family, and we refer to this *Ferrissia/Ancylus/Rhodacmea* clade as the subfamily Ancylinae. Interestingly, the Ancylinae appear to have experienced a series of genome duplication events because their collective chromosome complements form clean multiples: 15 (*Rhodacmea*), 30

(*Ferrissia*), and 60 (*Ancylus fluviatilis*) (Burch, 1960, 1962, 1965; Burch et al., 1960; Patterson and Burch, 1978).

Status of *Laevapex* nominal species

Although complicated by the presence of low frequency divergent mt lineages in some populations (discussed below), the molecular data, in addition to the morphometric analyses (Fig. 2.2), were consistent with the conclusion that the investigated populations of nominal *Laevapex* species represent a single polymorphic lineage. All of the nominal taxa (*L. fuscus*, *L. diaphanus*, *L. peninsulae*, *L.* sp., and "*F.*" *arkansasensis*) were polyphyletic in the mt gene trees (Fig. 2.4a), exhibited modest levels (3.9%) of genetic divergence in the primary (103/109 individuals) mt clade and, with one minor exception at the southern edge of their collective range, they appeared fixed for a single ITS-2 genotype (Fig. 2.4b).

The nominal species descriptions were found to be poor predictors of genealogical relationships, most emphatically in the case of putative "*F.*" *arkansasensis* (synonymized to *Ferrissia rivularis* by Basch [1963b]) that proved to be an unambiguous member of the *Laevapex* clade in our gene trees (Figs. 2.3, 2.4). The Oklahoma limpets were originally identified as *Hebetancylus excentricus* based on the absence of pigment in their tentacles (R. McMahon, pers. comm.), a feature that distinguished Floridian *Laevapex fuscus* from co-occurring *H. excentricus* specimens (Basch, 1963b) and that had been incorporated into freshwater snail identification keys (Basch, 1963b; Burch, 1982). However, *Laevapex* populations display considerable variation in tentacle pigmentation (Basch,

1963b; Walther, pers. observ.), and the Oklahoma specimens lack other, more definitive, *H. excentricus* characteristics such as a subacute, distinctly eccentric apex (Basch, 1963b; Burch, 1982; Thompson, 1999). Our gene trees indicate that the shell shape and sculptural features used to distinguish nominal *L. fuscus*, *L. diaphanus*, and *L. peninsulae* are unreliable phylogenetic indicators, and they presumably encompass a large ecophenotypic component (Basch, 1963a, 1963b; Durrant, 1975; Sutcliff and Durrant, 1977; McMahon and Whitehead, 1987; McMahon, 2004). Joint consideration of the gene tree and morphometric data lead us to conclude that there is but one species of *Laevapex* in eastern North America and that *L. diaphanus*, *L. peninsulae*, *L.* sp., and "*F.*" *arkansasensis* be collectively synonymized with the type species *L. fuscus*. A similar process of taxonomic rationalization may also be required for some North American nominal species of *Ferrissia*, *e.g.*, our gene trees (Figs. 2.3, 2.4a) imply that *F. parallela* represents either a recently speciated sister lineage or a lentic ecophenotype of *F. rivularis*.

Significance of the divergent *Laevapex* mt genotypes

The presence of within-population pronounced mitochondrial diversity is not unprecedented in freshwater snails. All three freshwater cerithioidean radiations (Lydeard et al., 2002) contain multiple examples of within-species mt non-monophyly (Lydeard et al., 1998; Minton and Lydeard, 2003; Dillon and Frankis, 2004; Glaubrecht and Köhler, 2004; Köhler et al., 2004; Wilson et al., 2004; Rintelen et al., 2005). There are numerous potential explanations for this condition, including the presence of cryptic species, doubly uniparental inheritance of mitochondria (DUI), nuclear sequences of

mitochondrial origin (Numts), retention of ancestral polymorphisms or secondary contact, and introgression among genetically distinct populations (Funk and Omland, 2003). Three of these five alternatives can be rejected for our study system on the basis of the available data. The almost complete lack of reciprocal genetic differentiation observed for our nuclear marker (Fig. 2.4) rules out the possibility that our nominal limpet taxa constitute a cryptic species complex (Pfenninger et al., 2003). Presence of multiple deletions in a basal clade of divergent genotypes (Fig. 2.4a) superficially resembles the classic Numts scenario in which the translocated nuclear copies experience slower rates of evolution and, sheltered from purifying selection, accumulate deleterious mutations (Sorenson and Fleisher, 1996). However, inferred deletions were not restricted to the divergent clade (Figs. 2.4a, 2.5), did not disrupt the inferred COI reading frame, and (most persuasively) there was no evidence in the directly sequenced chromatograms for multiple copies of COI, a hallmark of the Numts condition (Sorenson and Fleisher, 1996). The absence of heteroplasmic sequences, together with the prevalence of hermaphroditism in ancylids (Basch, 1963b), also rules out the remarkable DUI system found in many bivalve mollusks (Hoeh et al., 1991; Liu et al., 1996; Passamonti et al., 2003; Quesada et al., 2003).

Long-term persistence of ancestral population polymorphisms is expected to result in the presence of highly divergent clades throughout a species' geographic range and the occurrence of many lineages of intermediate levels of divergence (Neigel and Avise, 1986). On the other hand, mitochondrial introgression in a region of secondary contact should lead to the co-occurrence of divergent haplotypes within limited subsections of a

taxon's geographic range and to the absence of intermediate haplotypes (Avise et al., 1987). We observed a somewhat intermediate condition in the *Laevapex* gene tree where rare divergent haplotypes were present throughout the southern half of the continental range, and they were sufficiently diverse (4 genotypes formed 3 long stem branches) to collectively approximate the appearance of intermediacy (Fig. 2.4a). We were not able to clearly distinguish between these two potential mechanisms, and a likely contributing factor was our failure to collect the hypothesized neotropical sister lineage *Hebetancylus excentricus* (Basch, 1963b). Multiple sampling efforts by us and by Floridian colleagues did not encounter this taxon in southern Florida locations where it had been recorded 40 years previously (Basch, 1963b). It is hoped that future successful sampling efforts there, or elsewhere in the Neotropics, will allow a more definitive testing of the evolutionary origins of the rare, phylogenetically divergent *Laevapex fuscus* haplotypes.

Laevapex fuscus **phylogeography**

Two temporally distinct models have been proposed to account for the diversification of the temperate freshwater faunas of eastern North America: a Gulf Coast allopatric species model predicts relatively old speciation events driven by Late Miocene/Pliocene sea level fluctuations (Near et al., 2003); a Pleistocene speciation model (Miller, 1965) predicts evolutionarily recent cladogenesis in spatially fragmented glacial refugia. Neither model is applicable to our *Laevapex fuscus* dataset due to a lack of evidence for speciation events. The relative absence of phylogenetic definition (apart from the rare divergent haplotypes) shown by *L. fuscus* across its extensive North American range is consistent with Basch's (1963b) hypothesis that it stems from an evolutionarily recent neotropical

colonization of the continent, however, confirmation of this view awaits the identification of convincing neotropical sister taxa.

Many northern hemisphere temperate taxa show within-species phylogeographic structure consistent with Quaternary climatic oscillation-driven north/south range fluctuations, *e.g.*, southern genetic richness versus northern genetic purity and the presence of relatively deep genetic divergences in putative southern refugial regions (Hewitt, 2000, 2004). Our northernmost (Michigan and Minnesota) samples of *Laevapex fuscus* exhibited relatively reduced genetic diversity levels consistent with expectations of Holocene northward expansion (Table 2.3). By far the highest levels of regional genetic divergence occurred in the mid-Atlantic samples, a regional location south of the maximum extent of the Laurentide Ice Sheet during the last glacial maximum (Dyke, 2004) and therefore a prime candidate for identification as a *L. fuscus* refugium (Hewitt, 2004). However, directional gene flow estimates (Table 2.4) undermine this view because they indicate that this region may have served more as a recipient (3.50 immigrants/generation from Alabama populations) than a source ($<3.95 \times 10^{-16}$ immigrants/generation to all other regional populations) of continental haplotypic diversity, *i.e.*, it may represent a center of lineage overlap for *L. fuscus*, rather than the primary ice age refugium.

Our data hint at possible refugia in two other locations: Alabama and Arkansas/Oklahoma. Despite the relatively small sample (N=6) from Alabama populations, they also exhibited high collective genetic diversity (Table 2.3) and were by

far the most convincing putative regional source of migrants, according to directional gene flow estimates (Table 2.4). The phylogeographic exclusivity of the Arkansas/Oklahoma tip clade (Figs. 2.4a, 2.6), together with its relative lack of genetic connectivity to our other study populations (Table 2.4), imply that it may also represent a geographically distinct refugium, a finding congruent with the proposed White River drainage *Percina evides* refugium (Near et al., 2001). Surprisingly, our southernmost population sample in Florida was the least diverse (Table 2.3) and the least connected (Table 2.4), although we cannot rule out the possibility that this may be an artifact of limited sampling intensity.

The *Laevapex fuscus* mt COI crown clade exhibits pronounced haplotypic diversity coupled with relatively modest molecular divergence levels and a high degree of population differentiation; a pattern of genetic structuring that holds considerable phylogeographic potential. We hope to intensively genotype *Laevapex fuscus* populations in selected North American drainages, including additional Floridian locations, to more fully flesh out Quaternary phylogeographic relationships.

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Table 2.1. Collection localities, codes for *Laevapex* samples, genotype information (# individuals sequenced/# unique haplotypes obtained), and voucher specimen information (University of Michigan Museum of Zoology [UMMZ] number, unless otherwise noted).

Taxon	Locality [with coordinates if known]	Code	28S	COI	ITS2	Catalog #
Family Ancylidae						
Laevapex fuscus (Adams, 1841)	Lake May, Cass Co., MN, USA [47°5.0'N; 94°36.0'W]	MN	3/1	3/2		BellMNH17039
	Pickerel Lake, Washtenaw Co., MI, USA [42°24.7'N; 83°59.0'W]	MI	1/1	12/2	1/1	300206
	South Anna River, Hanover Co., VA, USA [37°47.0'N; 77°35.0'W]	VAS		9/6	2/1	300207
	Salkehatchie River, Barnwell Co., SC, USA [33°23.7'N; 81°40.2'W]	SCS		2/2	2/1	300208
	Tensaw River, Mobile Co., AL, USA [31°03.1'N; 87°95.9'W]	ALT		2/1	1/1	300209-300210
L. diaphanus (Haldeman, 1841)	New River, Giles Co., VA, USA [37°19.0'N; 80°38.7'W]	VAN	1/1	12/5	2/1	300211
	Sugar Creek, York/Lancaster Co., SC, USA [35°00.3'N; 80°91.4'W]	SCC		12/4	2/1	300212-300213
	Cahaba River (at Marvel Slab), Bibb Co., AL, USA [33°10.0'N; 87°01.7'W]	ALM	1/1	1/1		300214
	Cahaba River (at Booth's Ford), Shelby Co., AL, USA [33°11.1'N; 87°00.1'W]	ALB	1/1	1/1		300215
	Black Warrior River, Jefferson Co., AL, USA [33°43.6'N; 86°58.9'W]	ALW	1/1	2/1		300216
L. peninsulae (Pilsbry & Johnson, 1903)	Creek east of Astor Park, Marion Co., FL, USA [29°12.5'N; 81°30.5'W]	FLA	3/1	6/1		300217
	Big Cypress Bend, Florida State Park, Collier Co., FL, USA [25°56.5'N; 81°28.2'W]	FLB	1/1	1/1	1/1	300218
Laevapex sp.	Mountain Fork River, McCurtain Co., OK, USA [34°08.1'N; 94°42.7'W]	OK	3/1	7/2	2/1	300219-300220
"*Ferrissia*" *arkansasensis* (Walker, 1925)	White River, Washington Co., AR, USA [35°58.0'N; 93°59'W]	ARW	3/1	11/1	1/1	300221
	Cossatot River, Polk Co., AR, USA [34°22.8'N; 94°14.0'W]	ARC	4/1	28/8	3/1	300222-300223

Table 2.1 (cont.)

Taxon	Locality [with coordinates if known]	Code	28S	COI	ITS2	Catalog #
Ferrissia rivularis (Say, 1817)	AuSable River, Crawford Co., MI, USA [44°40.0'N; 84°37.5'W]		1/1	1/1	1/1	300224
	New River, Giles Co., VA, USA [37°19.0'N; 80°38.7'W]			1/1		300225
	Little Cahaba River, Bibb Co., AL, USA [33°03.3'N; 86°58.2'W]		1/1	2/1		300226
F. fragilis (Tryon, 1863)	Pickerel Lake, Washtenaw Co., MI, USA [42°24.7'N; 83°59.0'W]		3/1	1/1	2/1	300227
	Salkehatchie River, Barnwell Co., SC, USA [33°23.7'N; 81°40.2'W]		1/1	1/1		300228
	Cahaba River (at Booth's Ford), Shelby Co., AL, USA [33°11.1'N; 87°00.1'W]		1/1	2/1		300192
	Cahaba River (at CR 52 crossing), Shelby Co., AL, USA [33°17.0'N; 86°53.0'W]		1/1	2/2		300193
F. parallela (Haldeman, 1841)	Douglas Lake, Cheboygan Co., MI, USA [45°35.0'N; 84°40.0'W]		1/1	1/1	1/1	300229
Rhodacmea elatior (Anthony, 1855)	Cahaba River (at Marvel Slab), Bibb Co., AL, USA [33°10.0'N; 87°01.7'W]		1/1	1/1		300230
Uncancylus sp.	Parque Pereyra, Buenos Aires, Argentina		1/1	2/1	1/1	300231
Ancylus fluviatilis (Müller, 1774)	Abhainn an Chnoic, Indreabhán, Co. Galway, Ireland		2/1	2/1		300232
Family Acroloxidae						
Acroloxus lacustris (Linnaeus, 1758)	Leacht Lake, Moylough, Co. Galway, Ireland [53°29.9'N; 08°36.1'W]		2/1	2/2		300194
Acroloxus coloradensis (Henderson, 1930)	Lost Lake, Boulder Co., CO, USA [39°57.0'N; 105°37.3'W]		2/1	2/1		300195

Table 2.1 (cont.)

Taxon	Locality [with coordinates if known]	Code	28S	COI	ITS2	Catalog #
Family Planorbidae						
Gyraulus deflectus (Say, 1824)	Pickerel Lake, Washtenaw Co., MI, USA [42°24.7'N; 83°59.0'W]		1/1			300246
Family Lymnaeidae						
Stagnicola emarginata (Say, 1821)	Douglas Lake, Cheboygan Co., MI, USA [45°35.0'N; 84°40.0'W]		1/1			300245

Table 2.2. Diagnostic morphological characters and distributions of nominal *Laevapex* species. Unless otherwise noted, information in the table is from the original species description. Two nominal taxa included in the study (*Laevapex* sp. sampled from Oklahoma and *"Ferrissia" arkansasensis* sampled from Arkansas) do not appear in this table because of manifest uncertainty concerning their taxonomic status (see Materials and Methods for details).

Species	Aperture Shape	Radial sculpture	Average dimensions (length x width x height)	Range/habitat
Laevapex fuscus (Adams, 1841)	elliptical	smooth or with fine raised riblets on anterior slope (Basch, 1963)	7.9 x 5.6 x 1.3 mm	eastern North America/still water (e.g. river backwaters and lakes) or slow-flowing rivers (Basch, 1963)
L. diaphanus (Haldeman, 1841)	nearly circular	smooth, often encrusted with dark material (Basch, 1963)	5.5 x 4.5 x 2.0 mm	south-central and eastern USA/slow-flowing streams (Basch, 1963)
L. peninsulae (Pilsbry & Johnson, 1903)	broadly oval	close, fine, and conspicuous radial striae on entire surface	7.0 x 5.0 x 1.7 mm	Florida peninsula, south and east of Suwannee River system (Thompson, 1999)/creeks

Table 2.3. Haplotype (H) and nucleotide diversity (π) with sampling variance calculated for the crown mt COI *Laevapex* clade (excluding the 4 divergent haplotypes).

Populations	# of individuals sequenced	# of haplotypes obtained	Haplotype diversity (H)	Mean number of pairwise differences (π)
Arkansas & Oklahoma	43	8	0.817±0.028	2.932±1.567
Alabama	6	4	0.867±0.129	4.600±2.627
South Carolina & Virginia	33	16	0.928±0.026	11.248±5.238
Michigan & Minnesota	15	4	0.705±0.074	1.676±1.040
Florida	6	1		

Table 2.4. Maximum likelihood estimates of gene flow among populations of *Laevapex* nominal species based on COI sequences. The analysis was carried out using an unrestricted migration matrix model with variable subpopulation size. The ML estimates (90% profile confidence intervals) are shown for population sizes ($\Theta = 2\mu N$) and number of immigrants per generation ($2mN$), where N is the effective population size and μ is the mutation rate per generation per site. Column headings show source populations, and row headings show recipient populations.

Partition	Population	$\Theta = 2\mu N_f$	Migration rate ($2mN$)				
			MI & MN	AR & OK	SC & VA	AL	FL
Total	MI & MN	0.0012 (0.0008 – 0.0017)		1.56×10^{-16} (1.38×10^{-16} – 0.08)	2.75×10^{-12} (2.40×10^{-12} – 0.08)	2.47×10^{-16} (2.17×10^{-16} – 0.08)	1.53×10^{-16} (1.35×10^{-16} – 0.08)
	AR & OK	0.0029 (0.0023 – 0.0038)	0.15 (0.02 – 0.55)		9.93×10^{-16} (3.47×10^{-16} – 0.20)	3.95×10^{-16} (3.45×10^{-16} – 0.20)	3.95×10^{-16} (3.49×10^{-16} – 0.20)
	SC & VA	0.0175 (0.0129 – 0.0244)	9.66×10^{-15} (8.48×10^{-15} – 0.68)	3.73×10^{-15} (3.28×10^{-15} – 0.68)		**3.50** (0.34 – 6.19)	2.35×10^{-15} (2.08×10^{-15} – 0.68)
	AL	0.0036 (0.0022 – 0.0066)	0.57 (0.22 – 1.18)	4.85×10^{-16} (4.29×10^{-16} – 0.19)	1.56×10^{-15} (1.37×10^{-15} – 0.19)		5.08×10^{-16} (4.49×10^{-16} – 0.19)
	FL	0.0002 (0.0001 – 0.0044)	7.28×10^{-10} (6.37×10^{-10} – 9.10×10^{-10})	1.18×10^{-12} (1.03×10^{-12} – 0.04)	0.03 (3.47×10^{-3} – 0.12)	2.05×10^{-17} (1.82×10^{-17} – 0.04)	
			Cossatot R., AR	White R., AR	OK		
AR & OK	Cossatot R., AR	0.0013 (0.0010 – 0.0019)		1.95×10^{-16} (1.72×10^{-16} – 0.42)	**1.24** (0.48 – 2.56)		
	White R., AR	0.0011 (0.0007 – 0.0019)	1.49×10^{-16} (1.31×10^{-16} – 0.18)		0.13 (0.01 – 0.48)		
	OK	0.0012 (0.0007 – 0.0022)	2.88×10^{-16} (2.53×10^{-16} – 0.15)	1.00×10^{-12} (8.77×10^{-13} – 0.15)			
			New R., VA	South Anna R. VA	SC		
SC & VA	New R., VA	0.0014 (0.0007 – 0.0027)		0.59 (0.13 – 1.67)	2.04×10^{-16} (1.80×10^{-16} – 0.39)		
	S. Anna R., VA	0.0138 (0.0076 – 0.0255)	3.55×10^{-10} (3.10×10^{-10}– 0.34)		7.10×10^{-11} (6.21×10^{-11} – 0.34)		
	SC	0.0030 (0.0017 – 0.0058)	5.69×10^{-16} (5.01×10^{-16} – 0.55)	0.73 (0.15 – 2.37)			

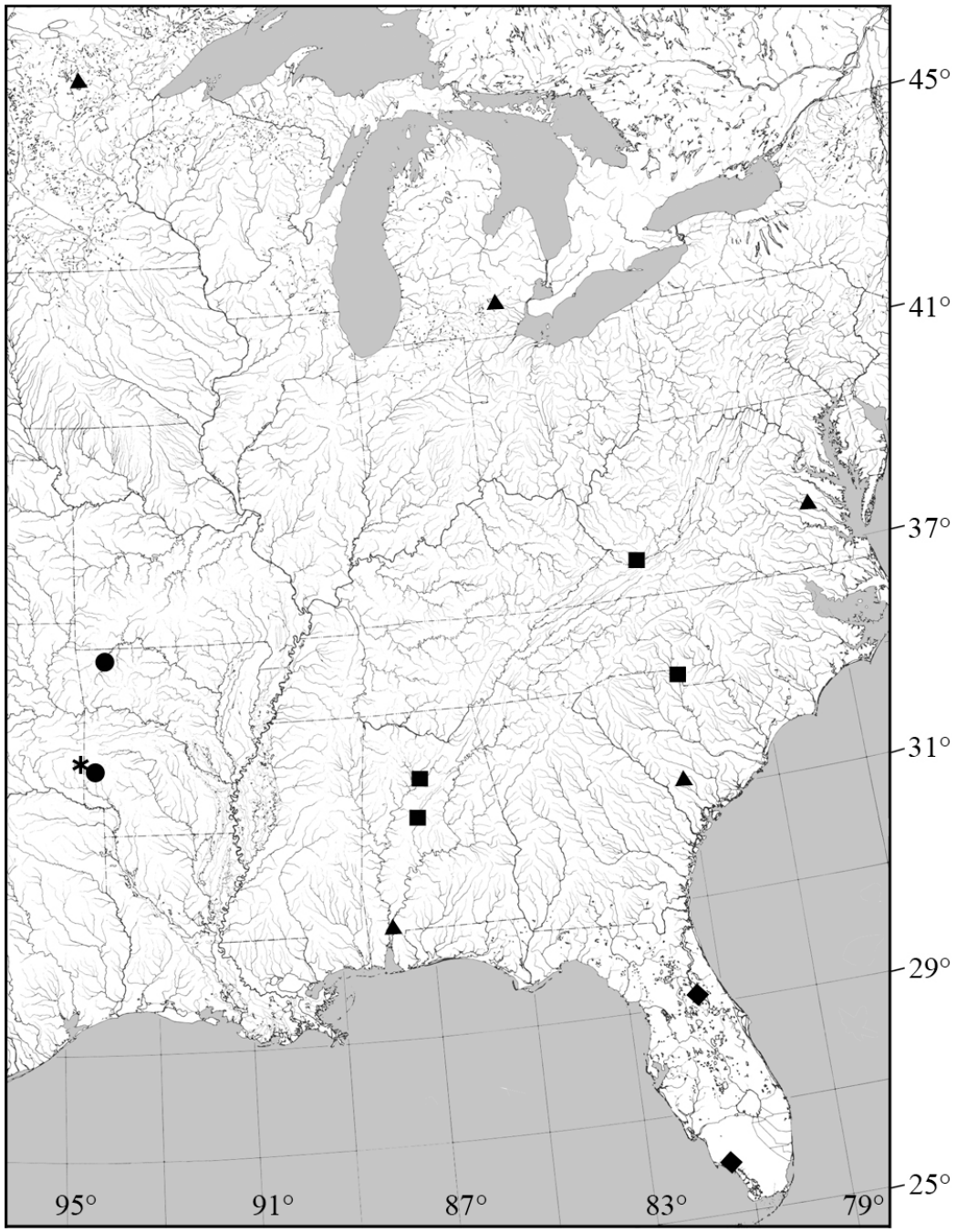


Figure 2.1. North American sampling localities for nominal *Laevapex* taxa: *L. fuscus* (▲), *L. diaphanus* (■), *L. peninsulae* (◆), *Laevapex* sp. (✻), and "*Ferrissia*" *arkansasensis* (●).

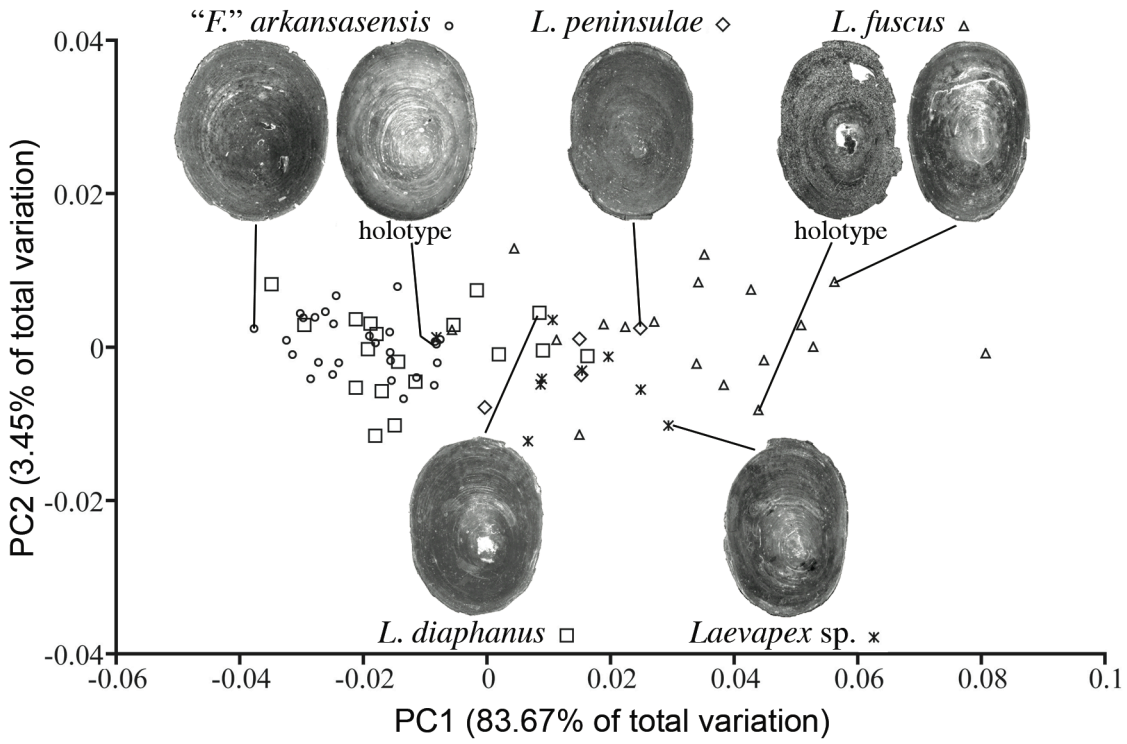


Figure 2.2. Principal component analysis (PCA) plot of apertural shape variation among nominal *Laevapex* taxa. Principal component 1 (PC1), accounting for almost all of the variation in shell apertural shape, summarizes the length to width ratio: limpets with higher scores are more elongate. PC2, a relatively negligible component of change, describes increasing anterio-lateral concavity and posterior convexity as scores increase. Shell shapes for individuals of each nominal taxon, and for two holotype specimens from the UMMZ collection (*L. fuscus* 68054(1); *F. arkansasensis* 100320), are shown. Note that none of the nominal taxa display morphospace exclusivity and that all of them overlap at PC1 values of -0.01 to 0.01.

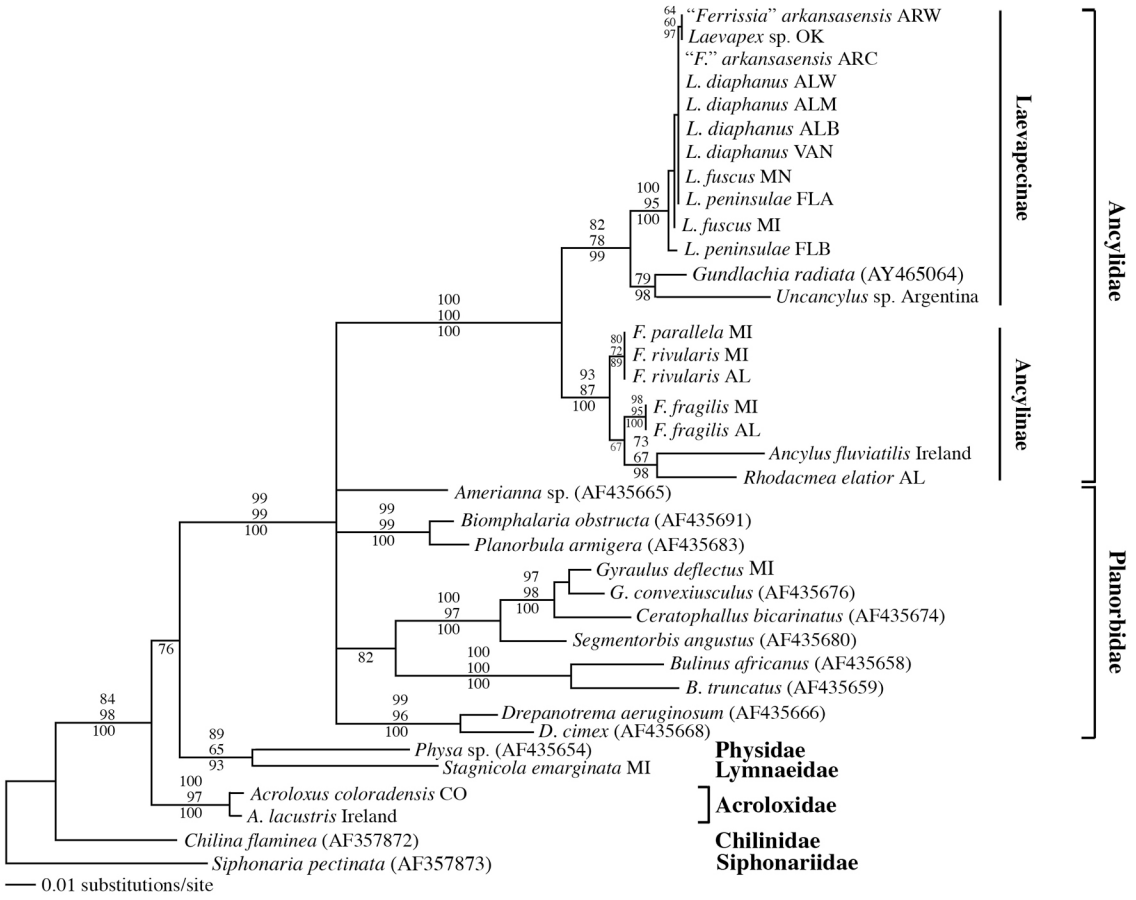
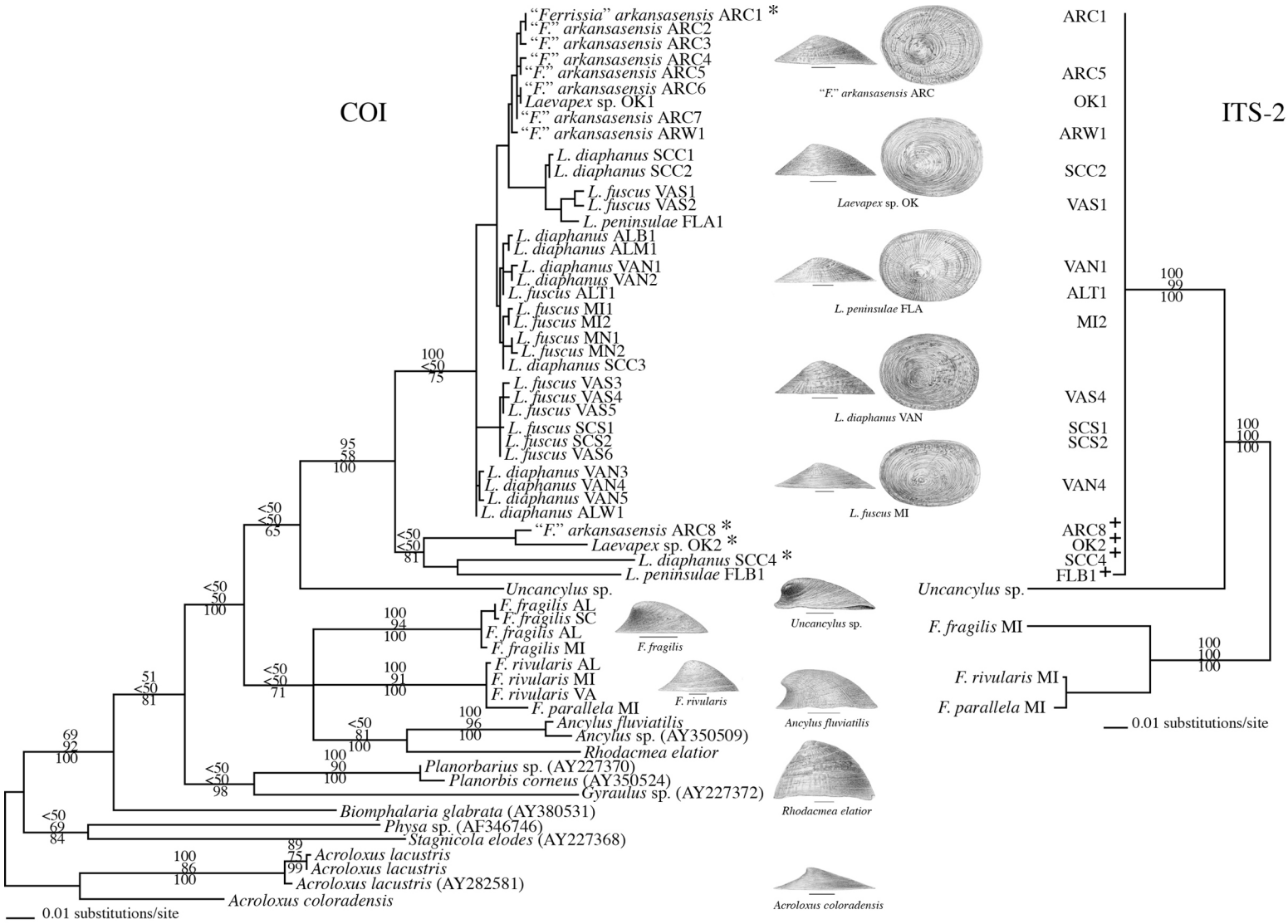


Figure 2.3. Bayesian phylogram for the nuclear 28S rDNA dataset, including 13 novel genotypes and 14 GenBank sequences. *Siphonaria pectinata* was the outgroup chosen to root the tree. Posterior probabilities are given below the branches. Maximum parsimony (top) and maximum likelihood (middle) bootstrap support values are also provided above the branches. See Table 2.1 for *Laevapex* nominal species location codes.

Figure 2.4. Bayesian phylograms for a) mt COI and b) nuclear ITS-2 datasets. The two *Acroloxus* species were set as the outgroups in a); the three *Ferrissia* species served as outgroups in b). Posterior probabilities are given below the branches. Maximum parsimony (top) and maximum likelihood (middle) bootstrap support values are provided above the branches. See Table 1 for location codes. The 4 mt COI haplotypes that contained inferred amino acid deletions are indicated by an * (a). ITS-2 nuclear genotypes of individuals containing divergent mt COI haplotypes (a) are indicated by an + (b). Drawings inserted between the gene trees depict freshwater limpet taxa included in the study. The scale bar shown below each shell profile represents 1 mm.


COI
"Ferrissia" arkansasensis ARC1 *
"F." arkansasensis ARC2
"F." arkansasensis ARC3
"F." arkansasensis ARC4
"F." arkansasensis ARC5
"F." arkansasensis ARC6
Laevapex sp. OK1
"F." arkansasensis ARC7
"F." arkansasensis ARW1
L. diaphanus SCC1
L. diaphanus SCC2
L. fuscus VAS1
L. fuscus VAS2
L. peninsulae FLA1
L. diaphanus ALB1
L. diaphanus ALM1
L. diaphanus VAN1
L. diaphanus VAN2
L. fuscus ALT1
L. fuscus MI1
L. fuscus MI2
L. fuscus MN1
L. fuscus MN2
L. diaphanus SCC3
L. fuscus VAS3
L. fuscus VAS4
L. fuscus VAS5
L. fuscus SCS1
L. fuscus SCS2
L. fuscus VAS6
L. diaphanus VAN3
L. diaphanus VAN4
L. diaphanus VAN5
L. diaphanus ALW1
"F." arkansasensis ARC8 *
Laevapex sp. OK2 *
L. diaphanus SCC4 *
L. peninsulae FLB1
Uncancylus sp.
F. fragilis AL
F. fragilis SC
F. fragilis AL
F. fragilis MI
F. rivularis AL
F. rivularis MI
F. rivularis VA
F. parallela MI
Ancylus fluviatilis
Ancylus sp. (AY350509)
Rhodacmea elatior
Planorbarius sp. (AY227370)
Planorbis corneus (AY350524)
Gyraulus sp. (AY227372)
Biomphalaria glabrata (AY380531)
Physa sp. (AF346746)
Stagnicola elodes (AY227368)
Acroloxus lacustris
Acroloxus lacustris
Acroloxus lacustris (AY282581)
Acroloxus coloradensis
0.01 substitutions/site
"F." arkansasensis ARC
Laevapex sp. OK
L. peninsulae FLA
L. diaphanus VAN
L. fuscus MI
F. fragilis
F. rivularis
Uncancylus sp.
Ancylus fluviatilis
Rhodacmea elatior
Acroloxus coloradensis
a)
ITS-2
ARC1
ARC5
OK1
ARW1
SCC2
VAS1
VAN1
ALT1
MI2
VAS4
SCS1
SCS2
VAN4
ARC8 +
OK2 +
SCC4 +
FLB1 +
Uncancylus sp.
F. fragilis MI
F. rivularis MI
F. parallela MI
0.01 substitutions/site
b)

"*Ferrissia*" *arkansasensis* ARC2	GVGTGWTVYPPLSGAVSHSGASVDLAIFSL
"*F.*" *arkansasensis* ARC1	GVGTGWTVYPPLSG-VSHSGASVDLAIFSL
"*F.*" *arkansasensis* ARC8	GVGTGWTVYPPLS--VY-SGTSVDLAIFSL
Laevapex sp. OK2	GVGTGWTVYPPLS--VY-SGTSVDLAIFSL
L. diaphanus SCC4	GVGTGWTVYPPLS--MTHS-ASVDLAIFSL
Uncancylus sp.	GVGTGWTVYPPLSGAVAHSGASVDLAIFSL
Ancylus fluviatilis	GAGTGWTVYPPLSGSIAHSGASVDLAIFSL
Acroloxus lacustris	GVGTGWTVYPPLSGPIAHAGASVDLAIFSL
Katharina tunicata	GAGTGWTVYPPLAGNVGHAGGSVDLAIFSL

Figure 2.5. Inferred translations of a 30 amino acid segment of mt COI (corresponding to nt positions 452 to 541 of the *Katharina tunicata* mt genome [Boore and Brown, 1994]) showing the *Laevapex* clade COI amino acid deletions encountered in this study, together with homologous sequences from other representative freshwater limpets. See Table 2.1 for location codes. Note that three distinct COI amino acid sequences were present in the Cossatot River, Arkansas (ARC) sample of 28 genotyped individuals.

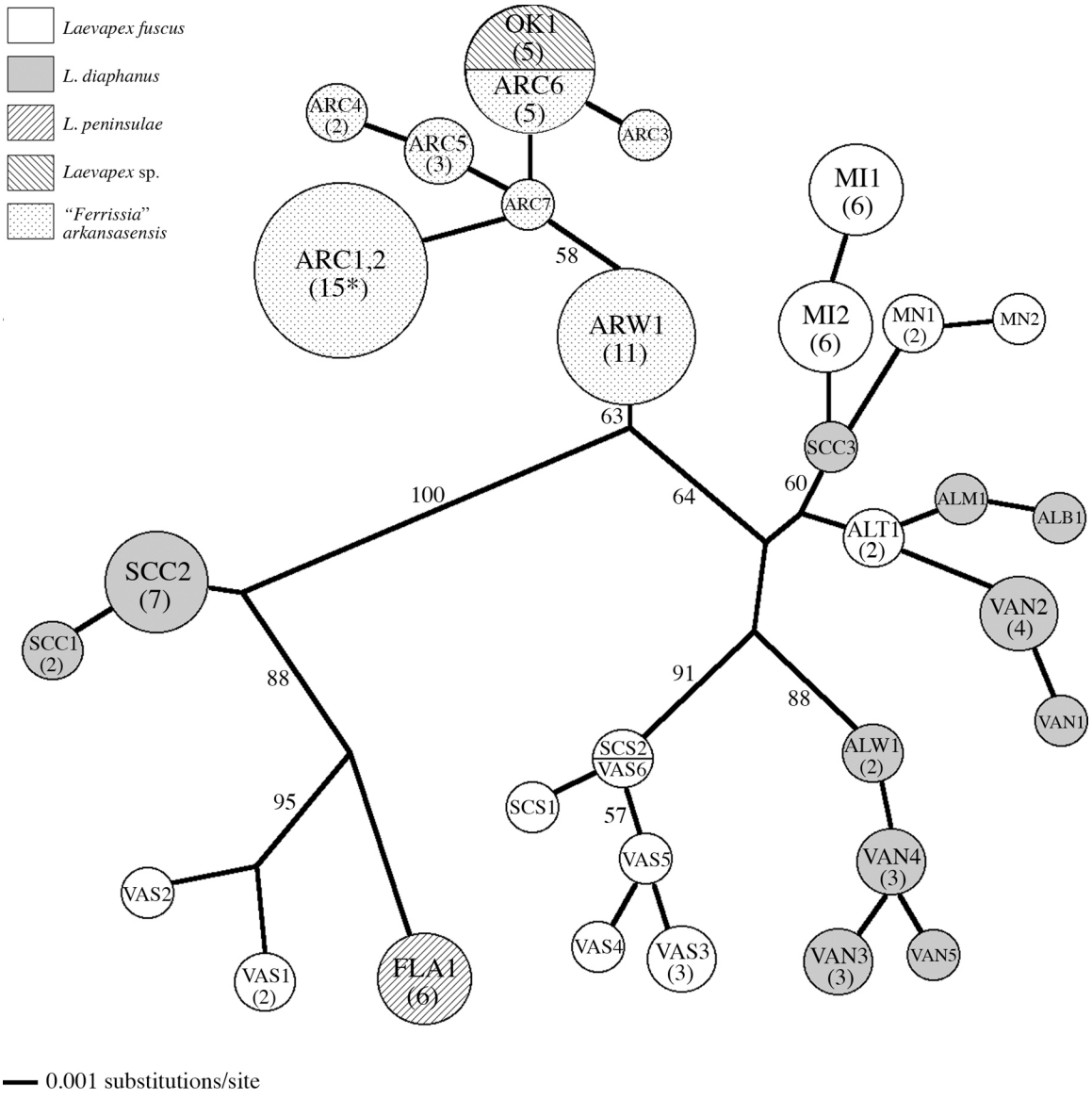


Figure 2.6. Maximum likelihood network for the *Laevapex* nominal species mt COI dataset (divergent haplotypes excluded). See Table 2.1 for location codes. Bootstrap values are provided along the branches. *Four of these 15 individuals sampled from the Cossatot River in Arkansas differed in that they had an inferred single amino acid (alanine) deletion.

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Chapter III

Attachment of the freshwater limpet *Laevapex fuscus* to the hemelytra of the water bug *Belostoma flumineum*

Abstract

Insects serve as agents of passive dispersal for freshwater limpets in the family Ancylidae. Previous studies of insect-mediated limpet dispersal have recorded limpets attaching themselves to beetles. We found the ancylid *Laevapex fuscus* attached to two individuals of the water bug *Belostoma flumineum* (Hemiptera: Belostomatidae) at a pond in Michigan in September 2006. Our discovery is the first description of belostomatids as a potential mode of transport for ancylids. Additionally, the timing of the observation suggests a possible seasonal component to such dissemination events; this association has never been observed in spring and summer surveys at the same site.

Understanding how organisms disperse to new habitats is a critical component of the study of ecological and evolutionary processes. The geographic distribution of freshwater mollusks demonstrates the importance of dispersal, as they can occur in small, isolated ponds, even those on remote oceanic islands (Baker, 1945). This phenomenon is explicable only when passive dispersal mechanisms are considered. Although birds are widely regarded as the primary non-human agent facilitating freshwater mollusk dissemination (Roscoe, 1955; Malone, 1965a, 1965b; Rees, 1965; Brönmark, 1985), limpets in one of the most ubiquitous freshwater snail families, the Ancylidae, exhibit a

unique mode of aerial dispersal. Ancylids crawl onto the wings of aquatic insects, often dytiscid and gyrinid beetles (Kew, 1893; Johnson, 1904; Rees, 1965; Rosewater, 1970), and are inadvertently transported as the insects travel from one freshwater habitat to another. Because ancylids are hermaphroditic and capable of self-fertilization (Basch, 1963), the introduction of a single individual into a new habitat is potentially sufficient for successful colonization.

It has been nearly four decades since the last account of ancylid attachment to an aquatic insect was published, largely because such occurrences are so rarely observed. Thus, little is known about the potential agents or timing of dispersal in ancylids. On 30 September 2006, we collected nine water bugs (Hemiptera: *Belostoma flumineum*) from Crane Pond (42°27'0.06"N, 84°00'52.49"W) at the University of Michigan's Edwin S. George Reserve (ESGR). Two of the *B. flumineum* had attached freshwater limpets. Each insect was carrying five limpets, identified as *Laevapex fuscus*, on its wings or hemelytra (Fig. 3.1). We are not aware of any other record of ancylid attachment to belostomatids. Given the positioning of the *L. fuscus* on the dorsal surfaces of the *B. flumineum*, it is uncertain if the insects were able to lift their hemelytra to take flight. Thus, whether a dispersal event was imminent in this case is unclear.

The association between *L. fuscus* and *B. flumineum* also appears to have a seasonal component. The ponds on the ESGR have been the focus of studies on the population dynamics of snails, insects, fish, and amphibians since 1996. Both *B. flumineum* and *L. fuscus* have been recorded at Crane Pond and other ponds each May and July since 1996,

but it was not until the fall of 2006 that *L. fuscus* was observed attached to *B. flumineum* (E. Werner and C. Davis, pers. comm.). Further investigation will be required to determine whether the behavior of either the insects or limpets changes seasonally, making contact between the two and potential aerial dispersal more likely during certain months. Other documented sightings of ancylid-insect associations exist for March (Rosewater, 1970), April (Johnson, 1904), May (Kew, 1893), and October (Johnson, 1904), though none of these records discusses implications of the timing of the finding.

The overall impact of insects on ancylid dispersal is unclear. While it has been suggested that insect vectors affect only the local distribution of ancylids (Rees, 1965), some have speculated that by "puddle-jumping," insects have impacted the large-scale geographic distribution of this taxon (Kew, 1893; Rosewater, 1970). Even if insect vectors influence only the local distribution of ancylids, this mode of dispersal still has significant implications in terms of population dynamics; increased colonization rates generally reduce species extinctions in metapopulations (Levins, 1970; Hanski and Gilpin, 1991). Thus, insect dispersal at least may help maintain local populations of ancylids. To better understand the role of various dispersal agents in ancylid dissemination, it is important that incidents of anyclid attachment to more mobile organisms continue to be documented.

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Figure 3.1. *Belostoma flumineum* with three *Laevapex fuscus* attached to its hemelytra. Two additional *L. fuscus* were removed from the wings before the photo was taken.

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Chapter IV

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

Freshwater biotas and ecosystems are being profoundly reshaped by ongoing human-mediated transoceanic biotic exchange (Benson, 1999). Although the establishment of invasive freshwater mollusc populations may involve highly conspicuous ecological perturbations (Nalepa and Schoesser, 1993; Darrigran and De Drago, 2000), 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 (Genner et al., 2004).

In a recent study (Walther et al., 2006a), 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) (Jørgensen et al., 2004), 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) (Boettger, 1949), a North American taxon later synonymized with *F. fragilis* (Basch, 1963). The mystery European limpet had been discovered a few years previously in southern France (Roger and Calas, 1944), and in the second half of the 20th

century it was sequentially recorded throughout much of the continent under various names (Wautier, 1974; Brown, 1977; Van der Velde and Roelofs, 1977; Strzelec, 2005). Taxonomic uncertainty pervades much of the freshwater limpet literature, due in part to pronounced ecophenotypic plasticity in shell morphology (Basch, 1963; McMahon and Whitehead, 1987). A wide diversity of opinion, in addition to Morrison's (Boettger, 1949), 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* (Calas, 1946; Hubendick, 1964, 1970; Wautier, 1974), described as a new species, *F. wautieri* (Mirolli, 1960), and synonymized with the Near Eastern/African limpet *F. clessiniana* (Jickeli, 1882) (Hubendick, 1970). In the recent European literature, it has been alternately referred to as either *F. wautieri* (Turner et al., 1998; Baur and Ringeis, 2002) or as *F. clessiniana* (Falkner and von Proschwitz, 1998; Strzelec, 2005), and each name is charged with distinct biogeographic associations. *F. 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*. In contrast, *F. clessiniana* is assumed to have recently spread across Western/Central/Eastern Europe from presumed endemic foci in either Southern Europe and/or North Africa (Falkner and von Proschwitz, 1998).

Based collectively on the phylogenetic placement of a voucher-less GenBank sequence (Walther et al., 2006a), Morrison's early (but overlooked) conchological identification (Boettger, 1949) and a number of striking ecological, morphological, and physiological

similarities (Walther et al., 2006a), 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* (Walther et al., 2006a). The 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 (Piechocki, 1986). In 2005, we obtained specimens sampled from ponds in Silesia, southern Poland (identified as *F. clessiniana*) (Strzelec, 2005), 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 analyzed together with homologous sequences from North American *F. fragilis*, Asian samples of the *Ferrissia* subgenus *Pettancylus* [diagnosed primarily on geographic grounds (Hubendick, 1964)], and available GenBank genotypes from other ancylid taxa. Molecular and phylogenetic techniques employed to amplify, sequence, and analyze the target gene fragments are detailed in two recent publications (Lee and Ó Foighil, 2005; Walther et al., 2006b), and novel sequences have been deposited in GenBank (28S: DQ452044-048; COI: DQ452031-035; 16S: DQ452036-043).

Figure 4.1 shows the gene tree topologies generated for the nuclear marker and mt 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 (Walther et al., 2006b). 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. 4.1). These globally distributed freshwater limpet specimens shared a single 28S genotype and displayed surprisingly modest levels of COI haplotypic diversity (≤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. 4.1). 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* (Basch, 1963), in these far-flung populations (Fig. 4.1). These include a very small (≤4 mm) and fragile shell 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 (Basch, 1963) that, as they diverge distally, accommodate new intervening striae (Burch, 1974).

Although the Philippine sample of nominal *Pettancylus* specimens was obtained from a single sampling site (E. Remigio, pers. comm.), 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 (4 inferred mutational steps), to an Argentine sample of *Uncancylus* sp. (Fig. 4.1). 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. 4.1) of a slender apex strongly hooked toward the right posterior side (Pilsbry, 1924) and a protoconch bearing a circular band of closely spaced microscopic shallow pits (Burch, 1974). These results indicate that the Philippines has experienced not one but two cryptic invasions of phylogenetically distinct New World ancylid taxa.

Although we were unable to confidently assign a species-level identity, the Philippine *Uncancylus* specimens appear to represent only the second record of the ancylid subfamily Laevapecinae outside of 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 (Brandt, 1974). 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 we document in the Philippines. Additionally, Brandt's description and photographic rendition of Thai specimens of another ancylid, *Ferrissia verruca* (Benson, 1855), looks suspiciously similar to *F. fragilis* (Brandt, 1974). 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 position within the *Uncancylus* and *F. fragilis* clades (Fig. 4.1).

Our novel 28S and mt COI results (Fig. 4.1) fulfil the phylogenetic prediction of the trans-Atlantic *Ferrissia fragilis* European invasion hypothesis (Walther et al., 2006a), 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 (Jørgensen et al., 2004; Walther et al., 2006a) and German (C. Albrecht unpubl.) *Ferrissia* specimens and also for a Ugandan *F. clessiniana* specimen (Jørgensen et al., 2004). We genotyped our Polish and North American samples of *F. fragilis* for mt 16S and phylogenetically analyzed them together with the supplementary sequences and a number of outgroup taxa (Fig. 4.2). 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. 4.2). The available data therefore corroborate the trans-Atlantic *F. fragilis* European invasion hypothesis (Walther et al., 2006a) and Morrison's initial identification (Boettger, 1949), although it is possible that phylogenetic characterization of southern European populations might eventually encounter *F. clessiniana* lineages (Falkner and von Proschwitz, 1998).

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 (Basch, 1963) underlay its pronounced invasiveness, though it is noteworthy that the first German (Boettger, 1949), Swedish (Falkner and von Proschwitz, 1998), and British (Brown, 1977) records were from artificial habitats (aquaria and botanical gardens). The potential presence of multiple inter-continental alien cryptic invaders complicates the study of an already challenging freshwater snail family, however, it is hoped that the increasing adaptation of molecular phylogenetic approaches will significantly enhance our understanding of ancylid systematics, ecology, and invasion history.

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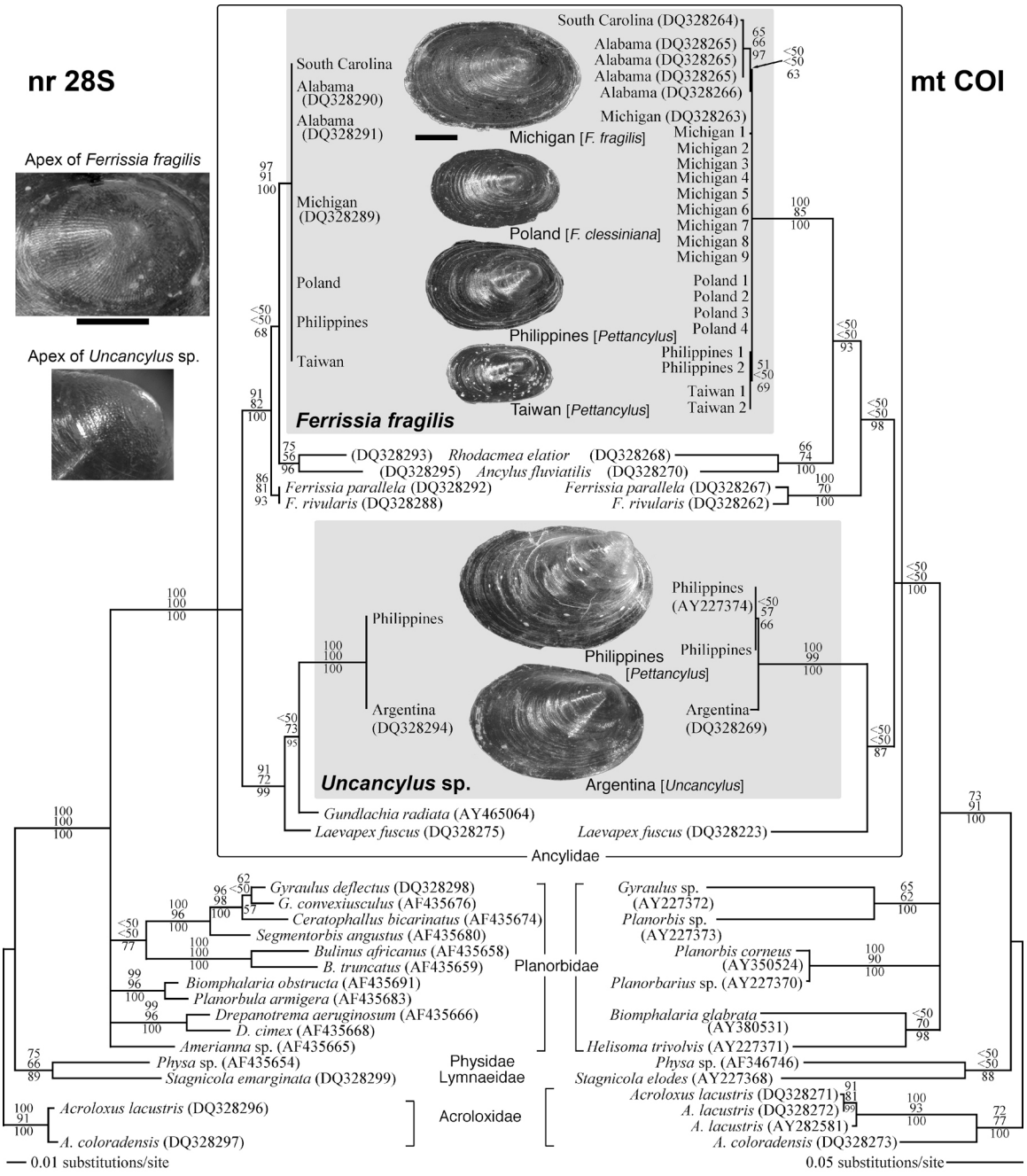
Table 4.1. Sampling locations for genotyped Polish and Asian limpets. Taxonomic designations assigned by the collectors.

Nominal taxon	Collector	Locality	UMMZ catalog number
Ferrissia clessiniana (Jickeli, 1882)	Strzelec	Upper Silesia, southern Poland	300279
Pettancylus sp.	Remigio	Alimodian, Iloilo Prov., Panay Is., Philippines	300276-277
Pettancylus sp.	Wu	Chi-Chi, Nantou Co., Taiwan	300278

Figure 4.1. Bayesian consensus phylograms for nuclear large subunit ribosomal DNA (nr 28S) and mitochondrial cytochrome oxidase I (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* (Remigio and Hebert, 2003). 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 (Basch, 1963; Burch, 1974) and for a Philippine *Uncancylus* sp. specimen showing the microscopic punctate protoconch sculpture typical of this genus (Burch, 1974).


nr 28S
mt COI
Apex of Ferrissia fragilis
Apex of Uncancylus sp.
South Carolina
Alabama (DQ328290)
Alabama (DQ328291)
Michigan (DQ328289)
Poland
Philippines
Taiwan
Ferrissia fragilis
South Carolina (DQ328264)
Alabama (DQ328265)
Alabama (DQ328265)
Alabama (DQ328265)
Alabama (DQ328266)
Michigan (DQ328263)
Michigan [F. fragilis]
Poland [F. clessiniana]
Philippines [Pettancylus]
Taiwan [Pettancylus]
Michigan 1
Michigan 2
Michigan 3
Michigan 4
Michigan 5
Michigan 6
Michigan 7
Michigan 8
Michigan 9
Poland 1
Poland 2
Poland 3
Poland 4
Philippines 1
Philippines 2
Taiwan 1
Taiwan 2
(DQ328293) Rhodacmea elatior (DQ328268)
(DQ328295) Ancylus fluviatilis (DQ328270)
Ferrissia parallela (DQ328292)
F. rivularis (DQ328288)
Ferrissia parallela (DQ328267)
F. rivularis (DQ328262)
Uncancylus sp.
Philippines
Argentina (DQ328294)
Philippines (AY227374)
Philippines [Pettancylus]
Argentina (DQ328269)
Argentina [Uncancylus]
Gundlachia radiata (AY465064)
Laevapex fuscus (DQ328275)
Laevapex fuscus (DQ328223)
Ancylidae
Gyraulus deflectus (DQ328298)
G. convexiusculus (AF435676)
Ceratophallus bicarinatus (AF435674)
Segmentorbis angustus (AF435680)
Bulinus africanus (AF435658)
B. truncatus (AF435659)
Biomphalaria obstructa (AF435691)
Planorbula armigera (AF435683)
Drepanotrema aeruginosum (AF435666)
D. cimex (AF435668)
Amerianna sp. (AF435665)
Planorbidae
Gyraulus sp. (AY227372)
Planorbis sp. (AY227373)
Planorbis corneus (AY350524)
Planorbarius sp. (AY227370)
Biomphalaria glabrata (AY380531)
Helisoma trivolvis (AY227371)
Physa sp. (AF435654)
Stagnicola emarginata (DQ328299)
Physidae
Lymnaeidae
Physa sp. (AF346746)
Stagnicola elodes (AY227368)
Acroloxus lacustris (DQ328296)
A. coloradensis (DQ328297)
Acroloxidae
Acroloxus lacustris (DQ328271)
A. lacustris (DQ328272)
A. lacustris (AY282581)
A. coloradensis (DQ328273)
0.01 substitutions/site
0.05 substitutions/site

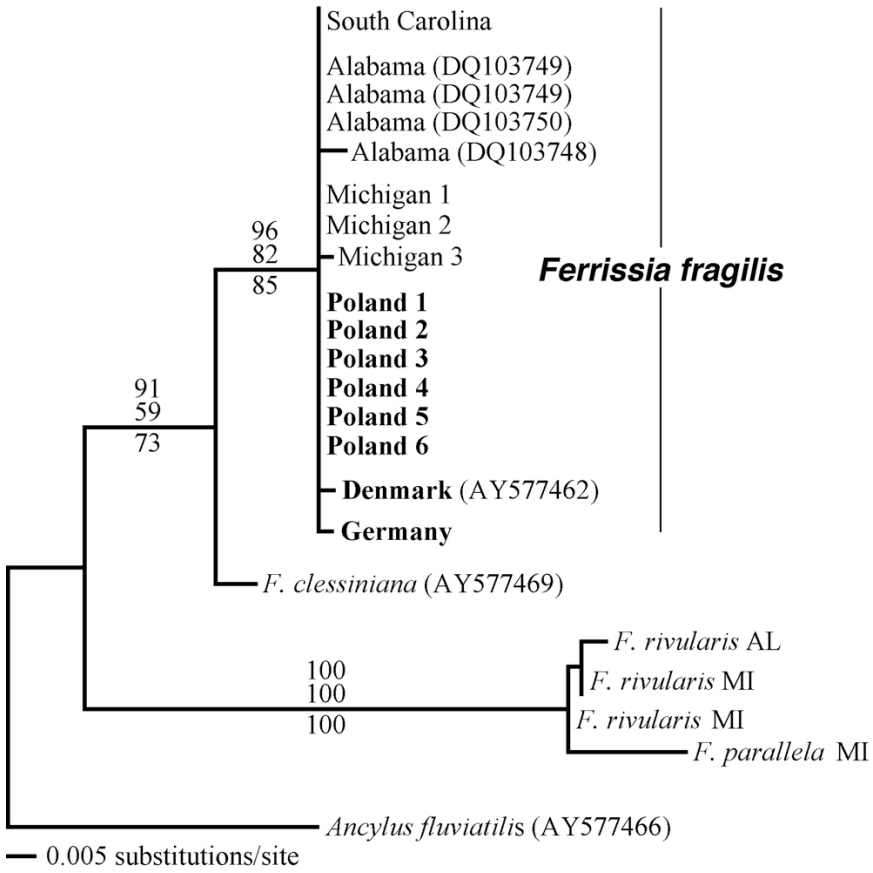


Figure 4.2. Bayesian phylogram of the ancylinid mitochondrial large subunit ribosomal DNA (mt 16S) dataset composed of four *Ferrissia* ingroup species and the designated outgroup *Ancylus fluviatilis*. Nodal posterior probabilities are given below, and maximum parsimony (top) and maximum likelihood (middle) bootstrap support values are presented above, the respective branches. GenBank accession numbers are given for non-novel haplotypes, and 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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Chapter V

A Systematic Review of North American *Ferrissia* (Hygrophila: Ancylidae)

Abstract

Members of the ancylid gastropod genus *Ferrissia* (Walker, 1903) have a near-cosmopolitan distribution in freshwater ecosystems but have received relatively little systematic attention in recent decades. In North America, the last major study was that of Paul Basch (1963), who recognized 5 *Ferrissia* species but stated *I have found no way to separate species unequivocally, and believe that they may all represent different directions of clinal variations within one large super-species*. Here I revisit the systematics of North American *Ferrissia* nominal taxa using molecular phylogenies to resolve taxonomic ambiguities. I obtained samples of the constituent nominal species from throughout their ranges in North America and utilized mitochondrial (mt) and nuclear markers to generate representative gene trees. My findings reveal two primary lineages of *Ferrissia* in North America. One *Ferrissia* lineage appears to be restricted to North America and is comprised exclusively of individuals from two nominal species: the lotic *F. rivularis* and the lentic *F. parallela*. These species are taxonomically polyphyletic, and the within-clade topology exhibits a pronounced geographic east/west structuring with evidence of limited secondary movement from east to west though no sign of introgression. The other primary North American lineage comprises *F. fragilis*, a minute pond-specialist capable of aestivating under drought conditions by producing a

septum. *F. fragilis* may have Old World sister lineages, and there is evidence of it being globally invasive, having established cryptic alien populations in tropical Asia and throughout Europe. It was first documented in Europe 60 years ago and has since been sequentially recorded across much of the continent as *F. wautieri* or *F. clessiniana*. Despite the number of *Ferrissia* sequences analyzed here, the monophyly of the genus remains uncertain. *Ferrissia* may well be paraphyletic, also containing *Ancylus* and *Rhodacmea* species, though more data is needed to definitively resolve this issue.

Introduction

Much of the world's freshwater malacofauna is under severe extinction pressure as a result of habitat degradation by damming and pollution (Neves et al., 1997), yet conservation efforts are hindered by the lack of systematic definition associated with many gastropod taxa. A recent increase in the use of advanced molecular techniques for freshwater snail studies is proving invaluable in resolving some lingering taxonomic uncertainties (Remigio and Hebert, 2003; Walther et al., 2006a; Albrecht et al., 2007; Wethington and Lydeard, 2007), but more gastropod taxa need to be revisited before we achieve a comprehensive understanding of the remaining biodiversity.

For over a century, taxonomic delineations and phylogenetic relationships in the widespread freshwater limpet family Ancylidae have remained largely ambiguous, and because ancylids exhibit pronounced ecophenotypic variation, considering morphological characters alone has confounded species-level assessments. Where molecular tools have already been utilized for ancylid research (Albrecht et al., 2004, 2007; Walther et al.

2006a, 2006b, 2006c) some long-accepted taxonomic distinctions have proven phylogenetically unsubstantiated and must be reevaluated. The ancylid genus *Ferrissia*, common in lentic and lotic freshwater habitats on every continent except Antarctica (Basch, 1963; Gómez et al., 2004), is among the most ubiquitous of all freshwater gastropods, making it especially prone to systematic confusion. *Ferrissia* species have been named and described around the world (Basch, 1963), yet without sufficient molecular data, it is unclear how many of the nominal taxa are valid and how they are related evolutionarily.

The most reliable diagnostic character of *Ferrissia* is a pattern of fine radiating grooves on the shell apex (Basch, 1963; Hubendick, 1964; Brandt, 1974; Burch, 1982), though this microsculpture, which originally develops on the embryonic shell, may be eroded in older specimens (Basch, 1963; Hubendick, 1964). Characteristic features of the soft anatomy of these minute (<1 cm long), hermaphroditic pulmonate snails include a flagellum-bearing simple penis (Hoff, 1940; Basch, 1963; Hubendick, 1964; Burch, 1982) and a single-lobed pseudobranch, or secondarily derived gill, that bears the anus (Hoff, 1940; Basch, 1963). Species delineations within *Ferrissia* have thus far been based exclusively on shell characters (e.g., length, thickness, aperture shape, the ability to form a septum) and the locality and habitat in which found (Table 5.1). As systematic studies continue to emphasize the disparity between morphological variation and underlying genetic variation in molluscan taxa, the need for a systematic review of *Ferrissia* has become evident.

Acknowledging the pronounced ecophenotypic plasticity exhibited by ancylids, Basch (1963) reduced over 20 nominal North American *Ferrissia* species to five (*F. rivularis*, *F. parallela*, *F. fragilis*, *F. walkeri,* and *F. mcneilli*) based strictly on morphological characters (Table 5.1). He admitted that some of these taxa are difficult to distinguish from others. Yet, since Basch's systematic revision, the group has been largely neglected, and taxonomic ambiguities have remained unresolved. Though, ultimately, a comprehensive systematic review needs to be completed for all nominal *Ferrissia* species from throughout their described ranges, this study aims to address Basch's work and resolve the systematic uncertainties associated with North American *Ferrissia*. Here, a molecular phylogenetic approach, in which both mitochondrial (mt) and nuclear markers were used, help reveal which of the nominal North American species are phylogenetically valid and how they are related to one another and to other ancylids.

Materials and Methods

Specimen collection

Nominal *Ferrissia rivularis*, *F. fragilis*, and *F. parallela* were collected from throughout their described ranges (Table 5.1). For the putative *F. mcneilli* and *F. walkeri*, both of which have restricted ranges, representative samples were collected near the respective type locality. Specimens collected in southwest MI, near the type locality of *F. michiganensis* (Winslow, 1923), a species Basch synonymized with *F. walkeri*, were included as an additional representative sample of *F. walkeri*. Collecting at the precise type localities of *F. mcneilli* and *F. walkeri* was not feasible for various reasons. The recorded type locality of *F. mcneilli* (Table 5.1), either no longer exists or the original

locality information was mislabeled (P. Johnson at the Alabama Aquatic Biodiversity Center, pers. comm.). Sampling the type locality described for *F. walkeri* was problematic because not only is the description vague, but many ponds in northern Arkansas are on private farms (D. Hayes at Arkansas State University, pers. comm.). Thus, even if the exact type locality of *F. walkeri* could be identified, it is likely inaccessible.

A few *Ferrissia* samples from outside of mainland North America were also collected and integrated into the study. A single specimen collected at the type locality stream near the type locality of the putative Hawaiian endemic *F. sharpi* (Sykes, 1900) was identified accordingly, and samples from France and the Azores were provided by generous colleagues who identified them as *F. clessiniana*. Specimens from the Philippines (*Pettancylus* sp.), Taiwan (*Pettancylus* sp.), and Poland (*F. clessiniana*) were first included in a previous ancylid study (Walther et al., 2006c), in which they were all found to be genetically indistinguishable from North American *F. fragilis*, but were revisited here using additional markers. Several non-*Ferrissia* ancylid samples from the eastern United States were also collected and included here. Table 5.2 provides the sampling and voucher information for all of the new samples represented in this study.

All specimens were preserved in 95% ethanol. Individuals were confidently identified as *Ferrissia* based on their minute size and radial apical microsculpture (Basch, 1963; Burch, 1988). Species identifications were assigned based on the descriptions available in Table

5.1. Admittedly, given the somewhat ambiguous species delineations within the genus, preliminary identifications to species were tenuous.

DNA sequencing

DNA was extracted and amplified closely following the steps outlined by Walther et al. (2006a). This study utilizes the same molecular markers: nuclear ribosomal large-subunit (28S), mt *cytochrome oxidase I* (COI), and nuclear second internal transcribed spacer (ITS-2). The only deviation from the protocol used in the earlier study was that, in some instances, the PCR product was purified directly using the Millipore Montáge PCR Cleanup Kit.

Phylogenetic analyses

Sequence chromatograms were edited in Sequence Navigator 1.0.1 (Applied Biosystems) by comparing the forward and reverse strands for each specimen. The revised sequences were then aligned for each gene separately using ClustalW (Thompson et al., 1994) implemented in Sequence Navigator and, where needed, the alignment was improved manually. In some cases, supplementary sequences were retrieved from GenBank and aligned with the novel genotypes. To further analyze phylogenetic relationships within the subfamily Ancylinae (*Ferrissia*, *Ancylus*, and *Rhodacmea*), a combined 28S+COI dataset, including only individuals that had been successfully sequenced for both genes, was constructed. The final aligned data matrices were 808 bp in length for 28S, 671 bp for COI, 639 bp for ITS-2 and 1424 bp for the concatenated 28S+COI dataset.

Sequences for each of the four datasets were assembled in a NEXUS format using Sequence Monkey 2.9.0. PAUP* 4.0b10 (Swofford, 2003) was used for phylogenetic analyses. A maximum parsimony (MP) analysis for each of the four datatsets was conducted using the heuristic search option (equal character weighting, 100 random stepwise additions, and tree-bisection-reconnection (TBR) branch-swapping). Inferred sequence gaps were coded as missing data. In the interest of time, the maximum number of trees saved for the COI and ITS-2 datasets was set to 5000. Bootstrap (Felsenstein, 1985) branch support (BS) levels were estimated with 100 replicates, 100 random additions each (only 10 random additions each in the MP COI analysis). No limit was set for the combined dataset MP bootstrap analysis, however, the maximum number of trees saved was set at 5000 for the other three datasets due to the lengthy computational time of bootstrap values. The nearest-neighbor interchange (NNI) branch-swapping algorithm was employed for generating ITS-2 bootstrap support, though TBR was used for the other three datasets. For maximum likelihood (ML) analyses, the best-fit model for each of the four datasets (TVM+I+G for 28S, COI, and the combined 28S+COI; TVM+I for ITS-2) was obtained in Modeltest 3.06 (Posada and Crandall, 1998). In each case, one of the MP trees was used as a starting tree with the TBR branch-swapping algorithm in PAUP*. Bootstrap support values were generated using a fast-heuristic search with 100 replicates. In MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003) Bayesian searches for each dataset were run for $1x10^6$ generations using the same best-fit model as in the ML analyses. Posterior probabilities were calculated by creating a majority-rule consensus for every 100^{th} tree after burn-in of the first 3000 trees using PAUP*.

Parsimony networks for the two major clades of North American *Ferrissia* were constructed using the statistical parsimony (Templeton et al., 1992) method in TCS 1.21 (Clement et al., 2000).

Results

Phylogenetic relationships of Ancylidae – 28S rDNA, mtCOI, combined 28S + COI

Figure 5.1 shows the Bayesian phylogram obtained for the 28S nuclear rDNA dataset including 28 GenBank sequences and sequences from 36 new samples. The MP and ML trees (not shown) generated for this dataset are similar in topology to the 28S phylogram, and any differences in tree topology among the analytical methods will be noted subsequently. Because all of the GenBank sequences incorporated into my 28S dataset were first analyzed together by Walther et al. (2006a), the 28S phylogram in Fig. 5.1 largely resembles that presented in the earlier study. The same outgroup (*Siphonaria pectinata*) is employed, and the same six families (Chilinidae, Acroloxidae, Lymnaeidae, Physidae, Planorbidae, and Ancylidae) make up the ingroup. Stem group relationships are as outlined by Walther et al. (2006a). The only stem node discrepancy among the three phylogenetic analyses is in the positioning of Acroloxidae with respect to the Lymnaeidae/Physidae clade. The acroloxids are basal to this clade for the ML and Bayesian analyses, though for MP they are weakly positioned sister to it.

As reported by Walther et al. (2006a), the 28S dataset also recovered a strongly (MP BS=100; ML BS=96; PP=100) monophyletic ancylid clade nested within the Planorbidae (Fig. 5.1), though a robust sister lineage to Ancylidae could not be identified. For the

Bayesian analysis, the ancylid clade is polytomous with the planorbid genera *Planorbula*, *Biomphalaria*, *Drepanotrema*, and *Amerianna*. Slightly more resolution is offered by the MP and ML analyses, in which the ancylids are sister to a *Planorbula*/*Biomphalaria* lineage and *Amerianna*, respectively; though again, bootstrap support values for these nodes are low (MP and ML BS<50).

The ancylids are separated into two robust clades, the Ancylinae and the Laevapecinae (Fig. 5.1), for all three 28S analyses. The Ancylinae contains three genera (*Ferrissia*, *Ancylus*, and *Rhodacmea*), with *Ferrissia* further subdivided into two distinct lineages in all three phylogenetic analyses. One *Ferrissia* lineage is comprised of all individuals identified as *F. rivularis* (the type species) or the nominal *F. parallela*, and the second contains those individuals identified as the nominal *F. fragilis*, *F. walkeri*, or *F. clessiniana*. My representatives of the putative *F. mcneilli* and the single Hawaiian *F. sharpi* were not successfully sequenced for 28S. All of the *F. rivularis* and *F. parallela* specimens share a single genotype for 28S, and the *F. walkeri* and *F. clessiniana* individuals sequenced share the most common *F. fragilis* 28S genotype; hence I designate the two lineages *F. rivularis* and *F. fragilis* (Fig. 5.1).

There is no evidence for a monophyletic *Ferrissia* for 28S, with an *Ancylus/Rhodacmea* clade tenuously (MP and ML BS<50; PP=61) sister to *F. fragilis* and *F. rivularis* sister to this clade. My mt COI analyses offer no resolve for this issue considering a substantially different topology was generated between the MP and the ML/Bayesian result. In one of 5000 MP COI trees presented (Fig. 5.2), *Rhodacmea* is weakly (BS<50) basal to *Ancylus*,

which is weakly (BS=55) basal to a monophyletic *Ferrissia* clade. Yet, for ML and Bayesian analyses performed on the same COI dataset, *Ancylus* and *Rhodacmea* form a well-supported clade (BS=80; PP=100) (Fig. 5.3) that, much like observed for 28S, nests within a paraphyletic *Ferrissia* lineage, weakly sister to *F. fragilis*. A combined 28S and COI gene tree generated to further assess support at the weaker nodes does not offer a convincing solution (Fig. 5.4). The *Ancylus*/*Rhodacmea* clade emerges in all three analyses with only moderate support values, and a monophyletic *Ferrissia* is poorly supported by all analytical methods.

The second major clade of ancylids, the Laevapecinae, was closely investigated by Walther et al. (2006a), though here I incorporate sequences from new samples into the 28S dataset, including representatives of *Laevapex*, *Hebetancylus excentricus*, and an unidentified Chilean specimen (CLL1). All new *Laevapex* nest neatly within a well-supported (MP BS=100; ML BS=87; PP=100) *L. fuscus* clade (Fig. 5.1). *H. excentricus* lies weakly (BS<50) sister to this clade for the MP analysis, though for the ML and Bayesian analyses *H. excentricus* is positioned sister to a *Gundlachia radiata*/*Uncancylus* sp./CLL1 clade. Depending on the analysis, the Chilean sample is either tenuously sister to *G. radiata* (as in MP and ML, BS<50 for both) or *Uncancylus* (as in Bayesian, PP=74). The interrelationships among the Laevapecinae are equally ambiguous for COI, and do not corroborate findings from 28S. Because I was unable to amplify the COI gene fragment for the Chilean specimen, this individual does not appear in the COI gene trees. While *H. excentricus* is robustly sister to a Costa Rican *G. radiata* (from a different population than the *G. radiata* included in the 28S analyses) for all three analytical

methods, the position of *L. fuscus* and *Uncancylus* sp. in relation to this clade is less stable. For MP, *Uncancylus* is weakly (BS<50) basal to *L. fuscus* which is weakly (BS=52) basal to the *H. excentricus*/*G. radiata* clade. For the ML and Bayesian phylogenies (Fig. 5.3), the *Hebtancylus*/*Gundlachia* clade is sister to *Uncancylus* with *L. fuscus* positioned basal to the neotropical taxa.

Phylogenetic status of nominal *Ferrissia* species – mt COI and nuclear ITS-2

COI and ITS-2 recovered some finer-scale genetic variation important in assessing species validity. While sequences from all five nominal North American *Ferrissia* species (plus *F. clessiniana* and *F. sharpi* individuals) are included in the COI and ITS-2 datasets, still only two major lineages of *Ferrissia* emerge in the representative gene trees (Figs. 5.2, 5.3, 5.5). As with 28S, one *Ferrissia* clade recovered by my COI and ITS-2 analyses contains sequences of individuals identified as *F. rivularis* and *F. parallela*. *F. parallela* is not genetically distinct from *F. rivularis*, however, as both taxa are polyphyletic for both markers; thus, this clade is identified as *F. rivularis*. Interestingly, the *F. rivularis* clade is subdivided into two distinct geographic lineages (differing by ≥16 steps) for COI (Fig. 5.2), one of which corresponds to samples from the western United States/Canada and the other comprised largely of individuals from the eastern United States. The one exception is that four individuals from a sampling site in Oregon are nested among the eastern haplotypes. My ITS-2 gene tree (Fig. 5.5) also shows some east-west geographic structure, though the eastern sequences occur as a polytomy along a branch shared with a robust western clade. The same four Oregonian specimens positioned in the eastern clade for COI also occur among the eastern sequences for ITS-2.

The second *Ferrissia* lineage contains sequences of the nominal *F. fragilis*, *F. walkeri, F. mcneilli*, *F. clessiniana*, and *F. sharpi*. *F. walkeri* and *F. mcneilli* are polyphyletic and nested within a paraphyletic *F. fragilis* for both markers (Figs. 5.2 and 5.5). For COI, haplotypes from individuals identified as *F. walkeri* and *F. mcneilli* differ from the most common *F. fragilis* haplotype by no more than two nucleotides each (Fig. 5.2); in a couple of instances they share haplotypes with individuals of *F. fragilis*. ITS-2 results (Fig. 5.5) corroborate this finding as specimens of the nominal *F. walkeri* and *F. mcneilli* share common *F. fragilis* ITS-2 sequences. Because these two putative taxa are not phylogenetically substantiated, the second *Ferrissia* clade is designated as *F. fragilis*. Interestingly, the Hawaiian specimen identified as the nominal *F. sharpi* positioned in the *F. fragilis* clade for COI and ITS-2 (Figs. 5.2 and 5.4) as well. This specimen shares a COI haplotype with several individuals from various localities in California. Azorean specimens initially identified as *F. clessiniana* nested within the *F. fragilis* clade for all three molecular markers used. While I did not amplify 28S and ITS-2 from the French *F. clessiniana* samples, I did find that four specimens from France share the most common *F. fragilis* COI haplotype. Additionally, Taiwanese and Philippine samples once identified as *Pettancylus* sp. until found to share COI haplotypes with North American *F. fragilis* (Walther et al., 2006c), were found to do the same here for ITS-2.

Three divergent (≥16 steps from the nearest haplotype) *F. fragilis* COI sequences (ALY1, ALY3, and FLM4) are clustered together in a small clade that stands out from the primary *F. fragilis* clade. For MP, this small clade is positioned basal to the larger (Fig. 5.2), though for the ML and Bayesian analyses the divergent haplotypes nest well within

the primary *F. fragilis* clade, coming off of a long branch (Fig. 5.3). An Australian *Ferrissia* sp. that was incorporated into the COI dataset gives a similar discrepant result for the different phylogenetic analyses. It emerges sister to the entire *F. fragilis* lineage for the MP analysis (Fig. 5.2), yet in the ML and Bayesian analyses, this individual appears as a long branch nested within the *F. fragilis* lineage (Fig. 5.3).

Shell vouchers from individuals sequenced for this study offer no foolproof characters for positively differentiating among members of the three different *Ferrissia* clades recovered by COI (Fig. 5.6). Shells from representatives of the *F. rivularis* eastern clade are as ecophenotypically variable as those from the western clade, and there is substantial overlap in shell sizes and shapes among members of the two clades. Differentiating between *F. rivularis* and *F. fragilis* is somewhat easier because *F. fragilis* do not grow as large as *F. rivularis*, though young or small *F. rivularis* may resemble adult *F. fragilis*. The apex of *F. fragilis* is generally located farther back and right of the midline than that of *F. rivularis*, but again, variation observed from one individual to another makes this character less reliable.

Discussion

Ancylid relationships

Seven widely accepted ancylid genera were represented in this study, making it the most comprehensive molecular systematic reassessment of the Ancylidae to date. My results corroborate previous evidence for two major findings: a monophyletic family Ancylidae nested within the higher limnic gastropod family Planorbidae (Albrecht et al., 2004;

Morgan et al., 2002; Remigio and Hebert, 2003; Walther et al., 2006a) and a pronounced sub-familial dichotomy within the Ancylidae (Albrecht et al., 2007; Walther et al., 2006a), which gives rise to a holarctic Ancylinae and a New World Laevapecinae (Walther et al., 2006a). Recently, it has been proposed that the family Ancylidae be reduced to subfamily status because of its position within the Planorbidae (Albrecht et al., 2007), though this change in taxonomic rank is largely inconsequential to my questions regarding evolutionary interrelationships within the Ancylidae.

The addition of novel sequences and ancylids from a broad range flesh out each of the two ancylid lineages better than previous studies, though some fundamental phylogenetic interrelationships within each clade remain unresolved due to still insufficient sampling. In the Ancylinae, nuclear and mitochondrial markers used here recover two distinct clades of *Ferrissia*, though my data confer no unambiguous support for this genus as a monophyletic group. *Ferrissia* may very well be paraphyletic, with an *Ancylus*/*Rhodacmea* clade sister to *F. fragilis*. Should this be the case, the *Ferrissia* condition may be plesiomorphic with *Rhodacmea* and *Ancylus* representing a more derived lineage. This is contrary to what some originally hypothesized regarding *Rhodacmea* having more primitive features than the other three North American genera (Burch, 1988), and it must be investigated further.

In the Laevapecinae, my *Laevapex* samples all fell neatly within a single well-supported *L. fuscus* clade, a finding that corroborates that of a previous study in which *L. fuscus* was deemed the only phylogenetically valid species of *Laevapex* (Walther et al., 2006a).

Hebetancylus excentricus from the U.S. appears in a molecular phylogeny for the first time here, emerging in a tip clade with a Mexican *H. excentricus* (sequence obtained from GenBank) in my COI gene trees, thus verifying that the identification of specimens from Alabama and Florida is correct. As would be expected, my *H. excentricus* samples do possess the telltale bi-lobed pseudobranch characteristic of seemingly all laevapecinids (Basch, 1963; Hubendick, 1964; Walther et al., 2006a). Having sequenced both nuclear 28S and COI for *Laevapex*, *Hebetancylus*, *Uncancylus*, and *Gundlachia*, however, does little to resolve phylogenetic relationships among these genera. Previous ancylid studies have recovered (*Laevpex* (*Gundlachia*, *Uncancylus*)) (Walther et al., 2006a) and (*Laevapex* (*Hebetancylus*, *Gundlachia*)) (Albrecht et al., 2007) sister relationships within this New World lineage, though when I combine these four taxa in a single study and add an unidentified Chilean specimen (in the 28S dataset only), relationships become tenuous and inconsistent between molecular markers and among phylogenetic analyses. Most of my results indicate that the neotropical genera form a single clade that is sister to *L. fuscus*, though a single exception presented here (Fig. 5.2) makes any definitive statement premature. As to the identity of the Chilean sample, it may well be a second species of *Uncancylus*, or it may belong to another laevapecinid genus of ancylids, *Anisancylus* (Pilsbry, 1924). Representatives of both of these genera have been recorded in Chile (Zarges, 2006). Unfortunately, I was unable to amplify COI for this specimen, which might have offered further insight into its identification. The neotropical clade within the Laevapecinae must be investigated further with a more expansive sampling from Central and South America to determine the precise number of genera and species and to resolve the interrelationships between these taxa.

Status of nominal Ferrissia species

The two major lineages of North American *Ferrissia* correspond to two species: *F. rivularis*, the type species, and *F. fragilis*, the second North American *Ferrissia* species described. Samples identified as the nominal *F. parallela* were genetically indistinguishable from *F. rivularis* for 28S and were polyphyletic for ITS-2 and COI, thus there is no molecular support for this taxon; it should be synonymized with the type species. Interestingly, this results means that *F. rivularis*, originally named for its widespread occurrence in rivers and streams, is not restricted to lotic environments after all and frequently occurs in lakes and ponds. Morphological characters attributed to *F. parallela*, including a narrow and more elongate shell (Table 5.1), were observed in some of my samples (Fig. 5.6), though more as a result of ecophenotypic variation (having been found on the stems of emergent pond vegetation) than any genetic predisposition. My finding supports an early statement by Basch (1963) indicating that *F. rivularis* and *F. parallela* are anatomically similar, though he still recognized them as distinct taxa.

Though there is no taxonomic structure in the *F. rivularis* clade, there is some east-west geographic structure, as recovered by COI and ITS-2 (Figs. 5.2 and 5.5). Thus, some level of divergence is occurring within this clade, though not in the north-south pattern anticipated by those who believed that *F. rivularis* occupied southern latitudes while *F. parallela* occupied more northern latitudes (Basch, 1963). Because specimens from Oregon share COI haplotypes and ITS-2 sequences with *F. rivularis* from the east, it appears that there is some movement of individuals from east to west, and there is no evidence for introgression. Thus, speciation may be occurring in the *F. rivularis* clade.

In the second major *Ferrissia* lineage, both the nominal *F. walkeri* and *F. mcneilli* lack phylogenetic support and appear to be synonyms of *F. fragilis*, assuming representative samples of the nominal species were collected (an inevitable limitation of my study). Unfortunately, specimens collected at the type locality of a taxon may not represent what occupied the habitat when a species was named and described from there, which is why I had to compare my shell vouchers to the shell of the holotype when available. The Hawaiian specimen identified as the putative Hawaiian endemic *F. sharpi* was no different than *F. fragilis* genetically (Figs. 5.2 and 5.5) or morphologically (Fig. 5.6), suggesting that *F. fragilis* invaded these islands over a hundred years ago, having since been mistaken for a native species. Similarly, specimens from France and the Azores identified as *F. clessiniana* are actually *F. fragilis*, which is no surprise given recent findings that *F. fragilis* is experiencing ongoing human-mediated transport and has cryptically invaded Europe and Asia (Walther et al., 2006b, 2006c).

My gene trees present significant variation between *F. rivularis* and *F. fragilis* and indicate that *F. fragilis* might have an Australian sister taxon. Also, *F. fragilis* may be more closely related to *Rhodacmea* and *Ancylus* than to its North American congener. Should it be determined that *F. fragilis* is distinct enough from *F. rivularis* to warrant being placed in a new genus, by the rules of scientific nomenclature, the genus name applied would have to be *Kincaidilla* (Hannibal, 1912); at one point, *F. fragilis* was thought to be a *Gundlachia* in the sub-genus *Kincaidilla*.

Here I have presented new, and likely unforeseen, insights into the diversity and

interrelationships of North American *Ferrissia*. Much like I observed in a similar study completed on *Laevapex* (Walther et al., 2006a), my latest findings clearly show how early malacologists frequently mistook ecophenotypic shell shape variation in ancylids for underlying genetic differences, naming and describing more species than molecular tools can account for. Thus, I reduce five nominal *Ferrissia* species down to two. On the other hand, some hidden genetic differences only become apparent after a molecular approach is used because shell and anatomical characters offer no clues. Differences between *F. rivularis* populations in the east and west, which might ultimately require that a new *Ferrissia* species be named, likely would have gone unnoticed without a thorough molecular phylogenetic reassessment of the genus. This study offers continued support for how a molecular approach is invaluable in modern-day systematic studies of freshwater snails.

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Table 5.1. Basic morphology and ecology of the five nominal species of North American *Ferrissia* recognized by Basch (1963). The information presented here was compiled from the original species descriptions, Basch (1963), and Burch (1982).

Nominal species	Shell length	Aperture shape	Shell thickness	Forms Septum	Habitat	Distribution	Type locality
F. rivularis (Say, 1817)	≤7 mm	oval, narrower at one end	robust	no	rivers and streams	across the United States	Delaware and Susquehanna rivers, PA
F. fragilis (Tryon, 1863)	≤4 mm	sides parallel or slightly incurved, ends rounded	very thin	yes	standing water	throughout the United States	Laguna Honda, CA
F. parallela (Haldeman, 1841)	≤9 mm	narrow, elongate, sides sub-rectilinear	thin	no	lakes	Canada and adjacent states	New England
F. walkeri (Pilsbry and Ferriss, 1907)	≤6 mm	oval, wider anteriorly	thin	no	ponds and lakes	Arkansas, Michigan, southern California	fish pond in Rogers, Benton Co., AR
F. mcneilli (Walker, 1925)	≤5 mm	oval, slightly wider anteriorly	thin	no	streams	southern Alabama, possibly Florida	Mandeville Creek, Mobile Co., AL

Table 5.2. Sampling localities, codes for samples, genotype information (# individuals sequenced/# unique genotypes obtained), and voucher information (University of Michigan Museum of Zoology [UMMZ] number, unless otherwise noted).

Taxon	Locality [with coordinates if known]	Code	28S	COI	ITS2	Catalog #
Family Ancylidae						
Ferrissia rivularis	Black River, northeast of Littlerock, Thurston Co., WA [46.9186°N; 123.0011°W]	WAB	-	2/1	1/1	301123
	Black River, just south of Mumby, Thurston Co., WA [46.8678°N; 123.0178°W]	WAC	1/1	3/2	1/1	301124
	Black Lake Ditch, west of Tumwater, Thurston Co., WA [47.0017°N; 122.9525°W]	WAD	-	2/1	1/1	301125
	Black River, Littlerock, Thurston Co., WA [46.9014°N; 123.0172°W]	WAL	-	4/1	1/1	301126
	small tributary (of Black River?), north of Littlerock, Thurston Co., WA [46.9186°N; 123.0178°W]	WAS	-	4/3	-	301127
	Battle Creek south of Salem, Marion Co., OR [44.8514°N; 1230517°W]	ORB	-	4/2	-	301128
	Mill Creek in The Dalles, Wasco Co., OR [45.6006°N; 121.1853°W]	ORL	3/1	12/3	6/2	301204, 301205
	Mill Creek, southwest of The Dalles, Wasco Co., OR [45.5678°N; 121.2342°W]	ORM	1/1	5/3	1/1	301129
	Pudding River, east of Salem, Marion Co., OR [44.9511°N; 122.8667°W]	ORP	-	4/2	-	301130
	Willamette River, southwest of Salem, Marion Co., OR [44.8675°N; 123.1353°W]	ORW	1/1	5/2	1/1	301131
	Feather River at Oroville Wildlife Area, south of Oroville, Butte Co., CA [39..4511°N; 121.6022°W]	CAF	1/1	1/1	2/1	301132
	MO, USA	MOA	-	-	5/1	301179
	Little River, Huntington, Huntington Co., IN [40.8681°N; 85.5011°W]	INL	1/1	1/1	1/1	301178
	AuSable River, Crawford Co., MI [44.6648°N; 84.6419°W]	MIA	1/1	1/1	1/1	300224

Table 5.2 (cont.)

Taxon	Locality [with coordinates if known]	Code	28S	COI	ITS2	Catalog #
***F. rivularis* (cont.)**	Fleming Creek, Washtenaw Co., MI [42.3050°N; 83.6617°W]	MIF	-	5/2	-	300280
	Little Cahaba River, Bibb Co., AL [33.0545°N; 86.9703°W]	ALK	1/1	2/1	-	300226
	Oaks Creek, Toddsville, Otsego Co., NY [42.8670°N; 74.9572°W]	NYO	-	2/2	2/1	301134
	Briar Creek, just west of Berwick, Columbia Co., PA [41.0456°N; 76.2851°W]	PAB	1/1	1/1	1/1	301133
	Susquehanna River, Watsontown, Northumberland Co., PA [41.0773°N; 76.8587°W]	PAS	1/1	1/1	1/1	301135
	New River, Giles Co., VA [37.3150°N; 80.6433°W]	VAN	-	1/1	-	300225
	Androscoggin River, West Bethel, Oxford Co., ME [44.4068°N; 70.8612°W]	MEA	-	4/2	-	301136
	Fish Stream, Patten, Penobscot Co., ME [45.9925°N; 68.4413°W]	MEF	-	1/1	1/1	301137
	Rockabema Stream, just southwest of Medway, Penobscot Co., ME [45.5946°N; 68.5482°W]	MEO	1/1	5/2	1/1	301138
	Rowe Brook, just west of Patten, Penobscot Co., ME [46.0013°N; 68.4784°W]	MER	1/1	3/2	1/1	301139
	Sweets Pond, Mansfield, Bristol Co., MA [41.9895°N; 71.2550°W]	MAS	1/1	-	-	301201
F. parallela	pond on North Pender Island, British Columbia, Canada [48.7358°N; 123.2356°W]	BC	3/1	1/1	1/1	301181
	Douglas Lake, Cheboygan Co., MI [45.5797°N; 84.6713°W]	MIL	1/1	1/1	1/1	300229
	Duck Island, Chippewa Co. [46.3667°N; 84.1347°W]	MID	-	4/1	-	301180
	Joe's Pond, West Danville, Caledonia Co., VT [44.4096°N; 72.2019°W]	VTJ	1/1	2/1	1/1	301140
	Kettle Pond, Lanesboro, Caledonia Co., VT [44.2964°N; 72.3077°W]	VTK	1/1	4/2	1/1	301141
	Beaver Dam Pond in Acadia National Park, Mt. Desert Island, Hancock Co., ME [44.3623°N; 68.1956°W]	MEB	1/1	4/1	-	301142
	Hamilton Pond, Mt. Desert Island, Hancock Co., ME [44.4273°N; 68.2843°W]	MEH	1/1	4/1	1/1	301143
F. fragilis	Edendale Creek, north of Merced, Merced Co., CA [37.4189°N; 120.5011°W]	CAE	-	4/2	-	301144

Table 5.2 (cont.)

Taxon	Locality [with coordinates if known]	Code	28S	COI	ITS2	Catalog #
***F. fragilis* (cont.)**	Merced River at Henderson Park, north of Merced, Merced Co., CA [37.5175°N; 120.4172°W]	CAM	1/1	4/2	1/1	301145
	Yosemite Lake, northeast of Merced, Merced Co., CA [37.3683°N; 120.4178°W]	CAY	-	5/3	-	301146
	Mexico, Veracruz, Xalapa, Jardin Botanico Clavijero	MXV	1/1	-	1/1	UGSB1206
	small creek above Burden Falls at Shawnee National Forest, IL [37.5634°N; 88.6422°W]	ILB	-	-	4/1	301182
	Pickerel Lake, Washtenaw Co., MI [42.4097°N; 83.9838°W]	MIP	1/1	10/2	2/1	300227, 301174
	Lake Maxinkuckee, Culver, IN [41.1839°N; 86.3850°W]	INM	1/1	-	-	301176
	Cahaba River at Booth's Ford, Shelby Co., AL [33.1853°N; 87.0015°W]	ALB	1/1	2/1	-	300192
	Cahaba River at CR 52 Crossing, Shelby Co., AL [33.2842°N; 86.8828°W]	ALC	-	2/2	-	300193
	Big Creek, south of Dothan, Houston Co., AL [31.0730°N; 85.4127°W]	ALA	-	2/2	2/1	301154
	Cahaba River, Centreville, Bibb Co., AL [32.9448°N; 87.1405°W]	ALD	-	1/1	1/1	301155
	Big Brush Creek, Wedgeworth, Hale Co., AL [32.8172°N; 87.7506°W]	ALG	-	2/1	2/2	301156
	Conecuh River, between Fairfield and Brooklyn, Covington Co., AL [31.1856°N; 86.6689°W]	ALH	-	2/1	2/2	301157
	Limestone Creek, Limestone Co., AL [34.6269°N; 86.8840°W]	ALL	-	1/1	1/1	301158
	Choctawhatchee River, Eunola, Geneva Co., AL [31.0294°N; 85.8538°W]	ALR	-	2/1	2/1	301159
	Yellow River, Covington Co., AL [31.3577°N; 86.3421°W]	ALY	-	3/3	2/1	301160
	Mulberry Fork of Black Warrior River, Bangor, Blount Co./Cullman Co., AL [33.9975°N; 86.7494°W]	ALZ	-	-	1/1	301161
	Lake Waccamaw, NC, USA [34.3066°N; 78.4957°W]	NCW	-	-	2/2	301175

Table 5.2 (cont.)

Taxon	Locality [with coordinates if known]	Code	28S	COI	ITS2	
***F. fragilis* (cont.)**	North Saluda River at Les Mullinax County Park, northeast of Lima, Greenville Co., SC [35.1271°N; 82.4264°W]	SCA	2/1	-	2/1	301187
	Potato Creek, southwest of Davis Station, Clarendon Co., SC [33.5735°N; 80.2926°W]	SCP	2/1	-	2/1	301183
	Salkehatchie River, Barnwell Co., SC [33.2370°N; 81.4020°W]	SCS	-	1/1	-	300228
	Laurel Lake, just west of Mattituck, Suffolk Co., NY [40.9778°N; 72.5562°W]	NYL	1/1	2/1	-	301147
	Lake Morey, Fairlee, Orange Co., VT [43.9264°N; 72.1417°W]	VTL	1/1	1/1	1/1	301148
	Mill Pond, Windsor, Windsor Co., VT [43.4761°N; 72.3976°W]	VTM	1/1	1/1	1/1	301149
	Sweets Pond, Mansfield, Bristol Co., MA [41.9895°N; 71.2550°W]	MAS	1/1	1/1	-	301200
	Quinebaug River, Plainfield, Windham Co., CT [41.7471°N; 71.9142°W]	CTQ	-	1/1	1/1	301150
	Penobscot River, northwest of Medway, Penobscot Co., ME [45.6149°N; 68.5392°W]	MEP	1/1	2/1	-	301151
	Little Falls Branch, MD [38.9441°N; 77.1169°W]	MDL	-	1/1	6/1	301186
	impoundment of North Mosquito Creek at Cypress Cove Nature Park, Gadsden Co., FL [30.7358°N; 84.8047°W]	FLM	-	1/1	1/1	301185
	subsidence pond in Upper Silesia, southern Poland	Poland	-	4/1	-	300279
	Chi-Chi, Nantou Co., Taiwan	Taiwan	-	2/1	2/1	300278
	Alimodian, Iloilo Prov., Panay Island, Philippines	Philip-pines	-	2/1	2/1	300276
F. mcneilli	Eslava Creek, Mobile Co., AL [30.6793°N; 88.1464°W]	ALE	-	1/1	1/1	301164
	Eslava Lake, Mobile Co., AL [30.6787°N; 88.1529°W]	ALF	-	1/1	1/1	301166
F. walkeri	small tributary entering Crystal Lake, Benton Co., AR [36.3352°N; 94.4338°W]	ARL	5/2	1/1	4/2	301193, 301194
	stream west of Harbert, Berrien Co., MI [41.8733°N; 86.6371°W]	MIW	-	3/1	5/1	301184
F. sharpi	Lulumahu Stream, Nuuanu Valley, Oahu, Honolulu Co., HI [21.3461°N; 157.8209°W]	HIO	-	1/1	1/1	301191
F. clessiniana	São Miguel Island, Azores	AZOR	1/1	1/1	1/1	301196
	Basin of Arcachon, south of Le Teich, France	France	-	4/1	-	301197

Table 5.2 (cont.)

Taxon	Locality [with coordinates if known]	Code	28S	COI	ITS2	Catalog #
Laevapex fuscus	Osage Creek, Benton Co., AR [36.1972°N; 94.3377°W]	ARO	1/1	-	-	301195
	marsh at the south end of Lake Maxinkuckee, Culver, IN [41.1839°N; 86.3850°W]	INM	2/1	-	-	301177
	Perry Lake at Perry Lakes Park, Marion, Perry Co., AL [32.6974°N; 87.2434°W]	ALP	1/1	-	-	301163
	Corn Creek Shoals, Coosa River, Elmore Co., AL [32.5519°N; 86.2011°W]	ALS	1/1	-	-	301198
	Pickerel Lake, Washtenaw Co., MI [42.4097°N; 83.9838°W]	MIP	1/1	6/1	1/1	300206
	Passaic River near Two Bridges, NJ [40.8858°N; 74.2689°W]	NJP	1/1	-	-	301152
	Wading River, Mansfield, MA [41.9894°N; 71.2548°W]	MAW	1/1	-	-	301153
	Big Cypress Bend, Florida State Park, Collier Co., FL, USA [25.9418°N; 81.4695°W]	FLB	1/1	-	-	300218
	Lake Helen (Helena), Volusia Co., FL [28.9850°N; 81.2303°W]	FLH	3/2	-	-	301189
	impoundment of North Mosquito Creek at Cypress Cove Nature Park, Gadsden Co., FL [30.7358°N; 84.8047°W]	FLM	6/1	-	-	301185
Hebetancylus excentricus	Eslava Creek, Mobile Co., AL [30.6793°N; 88.1464°W]	ALE	-	1/1	-	301165
	Eslava Lake, Mobile Co., AL [30.6787°N; 88.1529°W]	ALF	-	1/1	-	301167
	Lake Helen (Helena), Volusia Co., FL [28.9850°N; 81.2303°W]	FLH	2/1	1/1	-	301188
	Lake Talquin, west of Tallahassee, Leon Co., FL [30.4389°N; 84.5304°W]	FLT	6/1	-	-	301190
?	Lago Ranco, Chile [40.1910°S; 72.2606°W]	CLL	1/1	-	-	UGSB1201

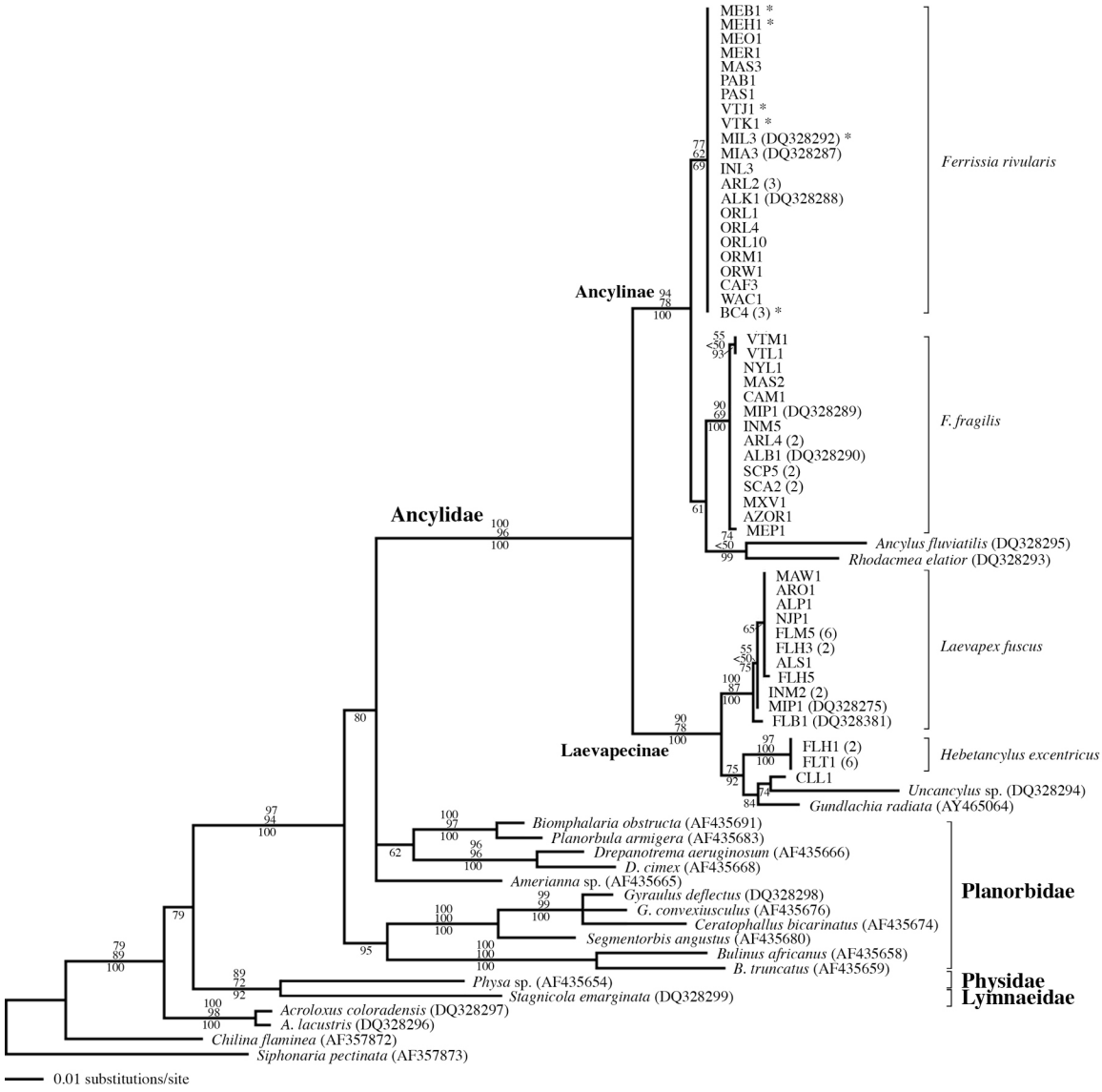
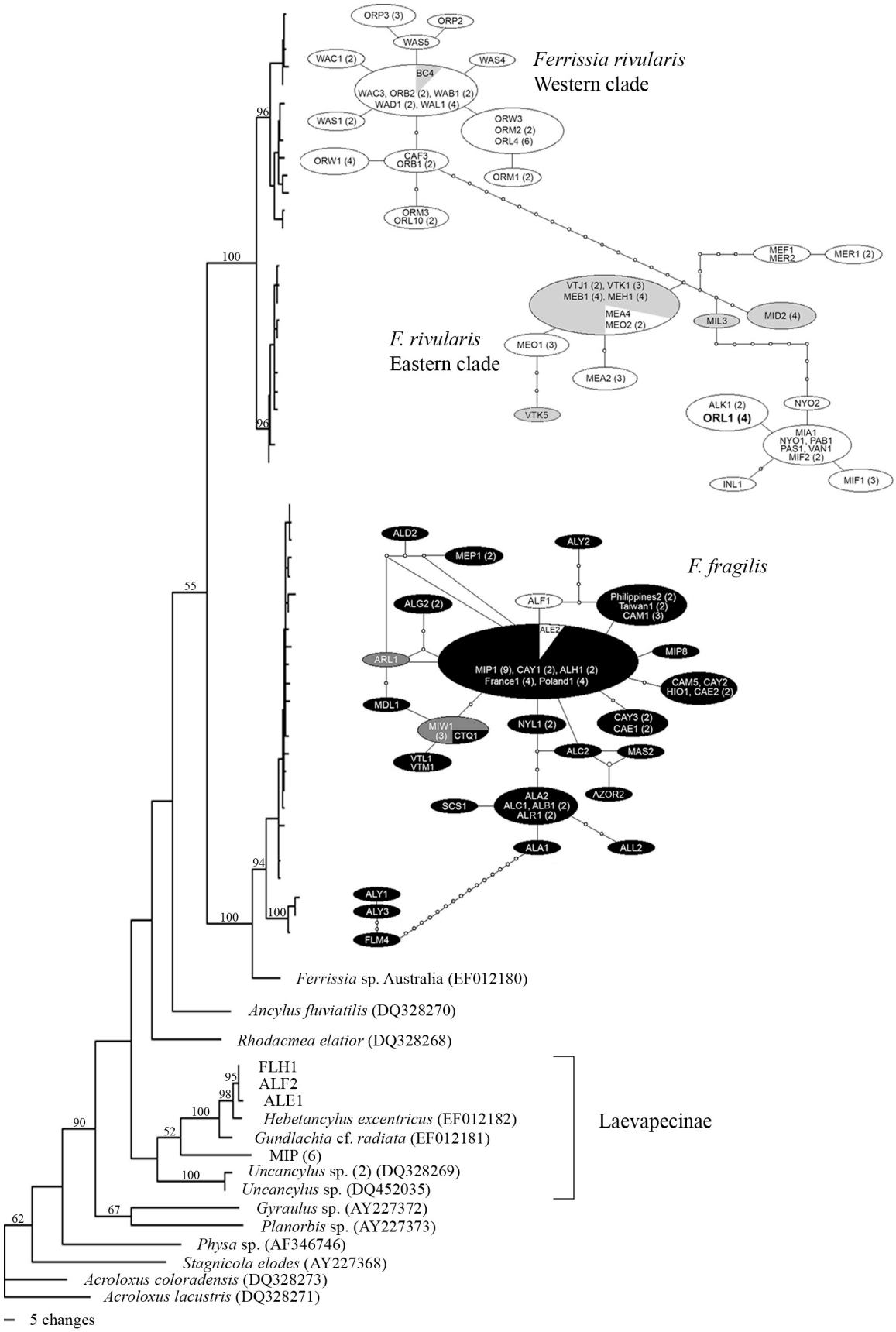


Figure 5.1. Bayesian phylogram for the nuclear 28S rDNA dataset, including genotypes from 36 new samples (locality codes provided in Table 5.2) and 28 GenBank sequences (GenBank numbers in parentheses). *Siphonaria pectinata* was designated as the outgroup. Bayesian posterior probabilities appear below the branches with maximum parsimony (top) and maximum likelihood (middle) bootstrap support values also presented. Where more than one specimen from a population share a genotype, the number of individuals sequenced is indicated as a single digit in parentheses. Individuals marked with an asterisk in the *Ferrissia rivularis* clade were originally identified as *F. parallela*.

Figure 5.2. One of 5000 most parsimonious trees for the mt COI dataset (strict consensus of 5000 trees differs only in the *Ferrissia* tip clades). Two *Acroloxus* species serve as the outgroup. Thirteen GenBank sequences appear in the tree (GenBank numbers in parentheses). Bootstrap support values are shown above the branches (omitted for tip clades). Unrooted *F. rivularis* and *F. fragilis* networks are used to show haplotype divergence among localities (locality codes provided in Table 5.2.). Single digit numbers in parentheses indicate how many individuals from a given population share a haplotype, and oval size corresponds to the total number of individuals sharing a haplotype. Oval colors represent original nominal species identifications. In the *F. rivularis* network, samples on a white background were identified as *F. rivularis*, while those on a shaded background were identified as *F. parallela*. In the *F. fragilis* network, individuals on a black background were identified as *F. fragilis*, those on a shaded background were identified as *F. walkeri*, and those on a white background were identified as *F. mcneilli*. ORL1 (4) is in bold because those four specimens were collected in the west but share an *F. rivularis* eastern clade haplotype.


ORP3 (3)
ORP2
WAS5
WAC1 (2)
WAS4
BC4
WAC3, ORB2 (2), WAB1 (2)
WAD1 (2), WAL1 (4)
WAS1 (2)
ORW3
ORM2 (2)
ORL4 (6)
ORW1 (4)
CAF3
ORB1 (2)
ORM1 (2)
ORM3
ORL10 (2)
Ferrissia rivularis
Western clade
96
100
MEF1
MER2
MER1 (2)
VTJ1 (2), VTK1 (3)
MEB1 (4), MEH1 (4)
MEA4
MEO2 (2)
MIL3
MID2 (4)
F. rivularis
Eastern clade
MEO1 (3)
MEA2 (3)
VTK5
ALK1 (2)
ORL1 (4)
NYO2
MIA1
NYO1, PAB1
PAS1, VAN1
MIF2 (2)
INL1
MIF1 (3)
96
F. fragilis
ALD2
MEP1 (2)
ALY2
ALF1
Philippines2 (2)
Taiwan1 (2)
CAM1 (3)
ALG2 (2)
ALE2
MIP8
ARL1
MIP1 (9), CAY1 (2), ALH1 (2)
France1 (4), Poland1 (4)
CAM5, CAY2
HIO1, CAE2 (2)
MDL1
MIW1
(3)
CTQ1
NYL1 (2)
CAY3 (2)
CAE1 (2)
VTL1
VTM1
ALC2
MAS2
AZOR2
SCS1
ALA2
ALC1, ALB1 (2)
ALR1 (2)
ALA1
ALL2
ALY1
ALY3
FLM4
55
94
100
100
Ferrissia sp. Australia (EF012180)
Ancylus fluviatilis (DQ328270)
Rhodacmea elatior (DQ328268)
FLH1
ALF2
ALE1
Hebetancylus excentricus (EF012182)
Gundlachia cf. radiata (EF012181)
MIP (6)
Uncancylus sp. (2) (DQ328269)
Uncancylus sp. (DQ452035)
Laevapecinae
95
98
100
52
100
90
67
62
Gyraulus sp. (AY227372)
Planorbis sp. (AY227373)
Physa sp. (AF346746)
Stagnicola elodes (AY227368)
Acroloxus coloradensis (DQ328273)
Acroloxus lacustris (DQ328271)
5 changes

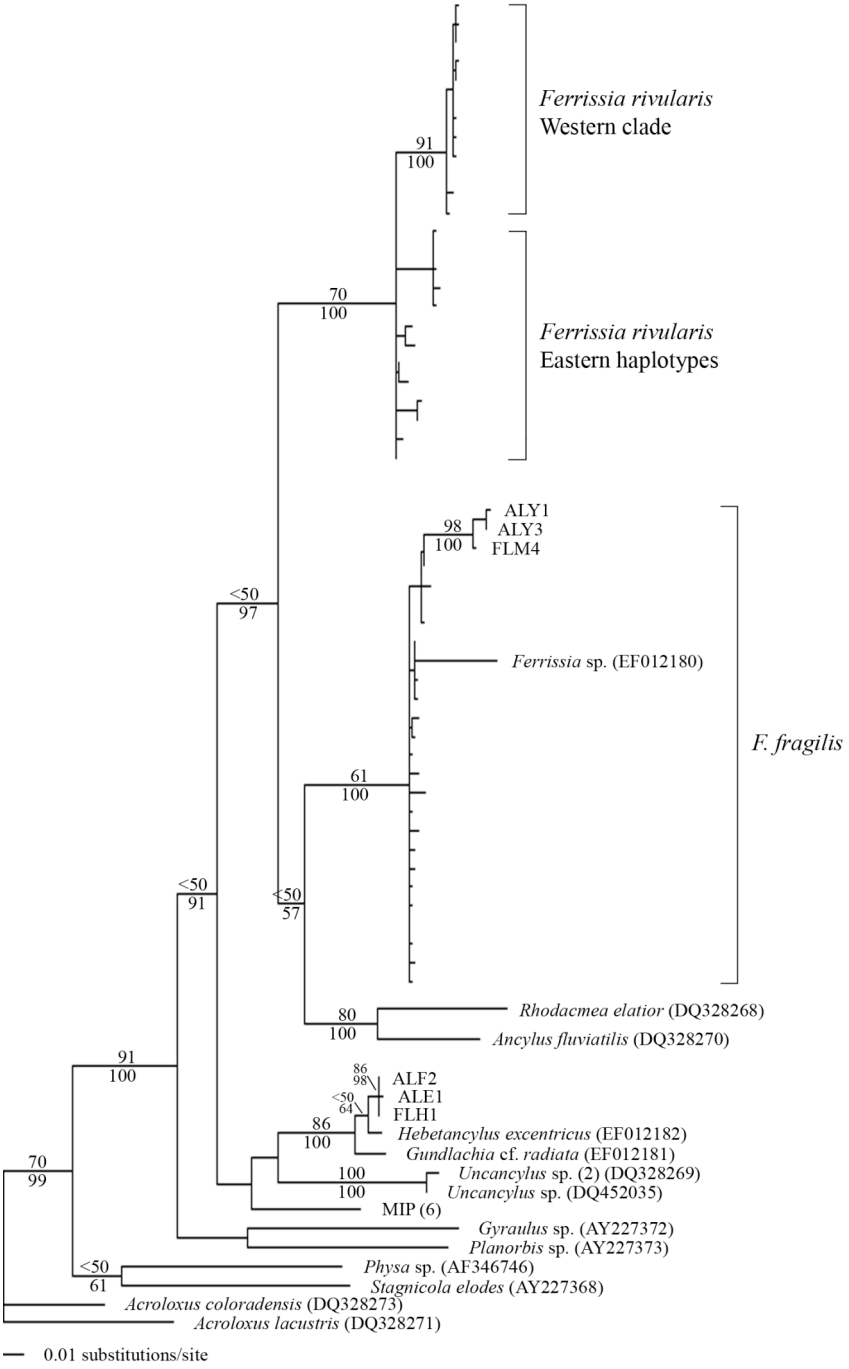


Figure 5.3. Bayesian phylogram for the mt COI dataset. Two *Acroloxus* species were selected as the outgroup. Thirteen GenBank sequences appear in the tree (GenBank numbers in parentheses). Locality codes for new samples are provided in Table 5.2. Where more than one specimen from a population share a haplotype, the number of individuals sequenced is indicated as a single digit in parentheses. Bayesian posterior probabilities appear below the branches with maximum likelihood bootstrap support values appearing above the branches.

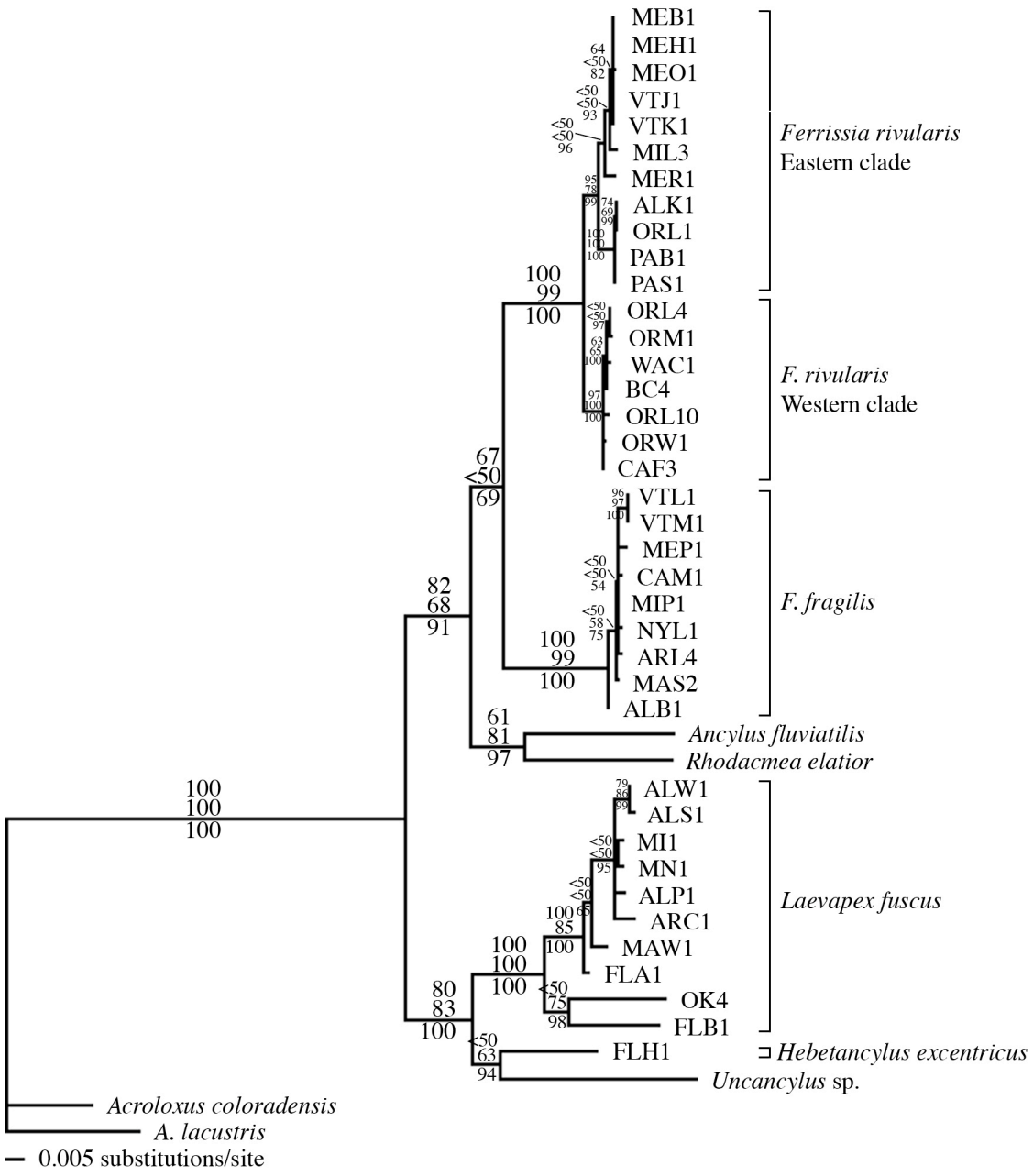


Figure 5.4. Bayesian phylogram generated for the combined nuclear 28S rDNA and mt COI dataset. The two *Acroloxus* species serve as the outgroup. Bayesian posterior probabilities appear below the branches with maximum parsimony (top) and maximum likelihood (middle) bootstrap support values also presented. Locality codes are provided in Table 5.2.

Figure 5.5. Bayesian phylogram for the nuclear second internal transcribed spacer (ITS-2) dataset. *Laevapex fuscus* was designated as the outgroup (GenBank number provided in parentheses). Bayesian posterior probabilities appear below the branches with maximum parsimony (top) and maximum likelihood (middle) bootstrap support values also presented. Locality codes are presented in Table 5.2, and where more than one specimen from a population share a genotype, the number of individuals sequenced is indicated as a single digit in parentheses. Samples in the *F. rivularis* clade that were originally identified as *F. parallela* are marked with an asterisk. Four Oregonian specimens that share an *F. rivularis* eastern clade genotype are in bold. In the *F. fragilis* clade, MIW1 (5) and ARL1 are representatives of *F. walkeri*, and ALE2 and ALF1 are representatives of *F. mcneilli*. HIO1 is the putative *F. sharpi* and AZOR2 is the putative *F. clessiniana*. The Philippine and Taiwan samples, formerly identified as *Pettancylus* sp. though showing *F. fragilis* affinities for COI (Walther et al., 2006c; present study), also nest within *F. fragilis* for ITS-2.

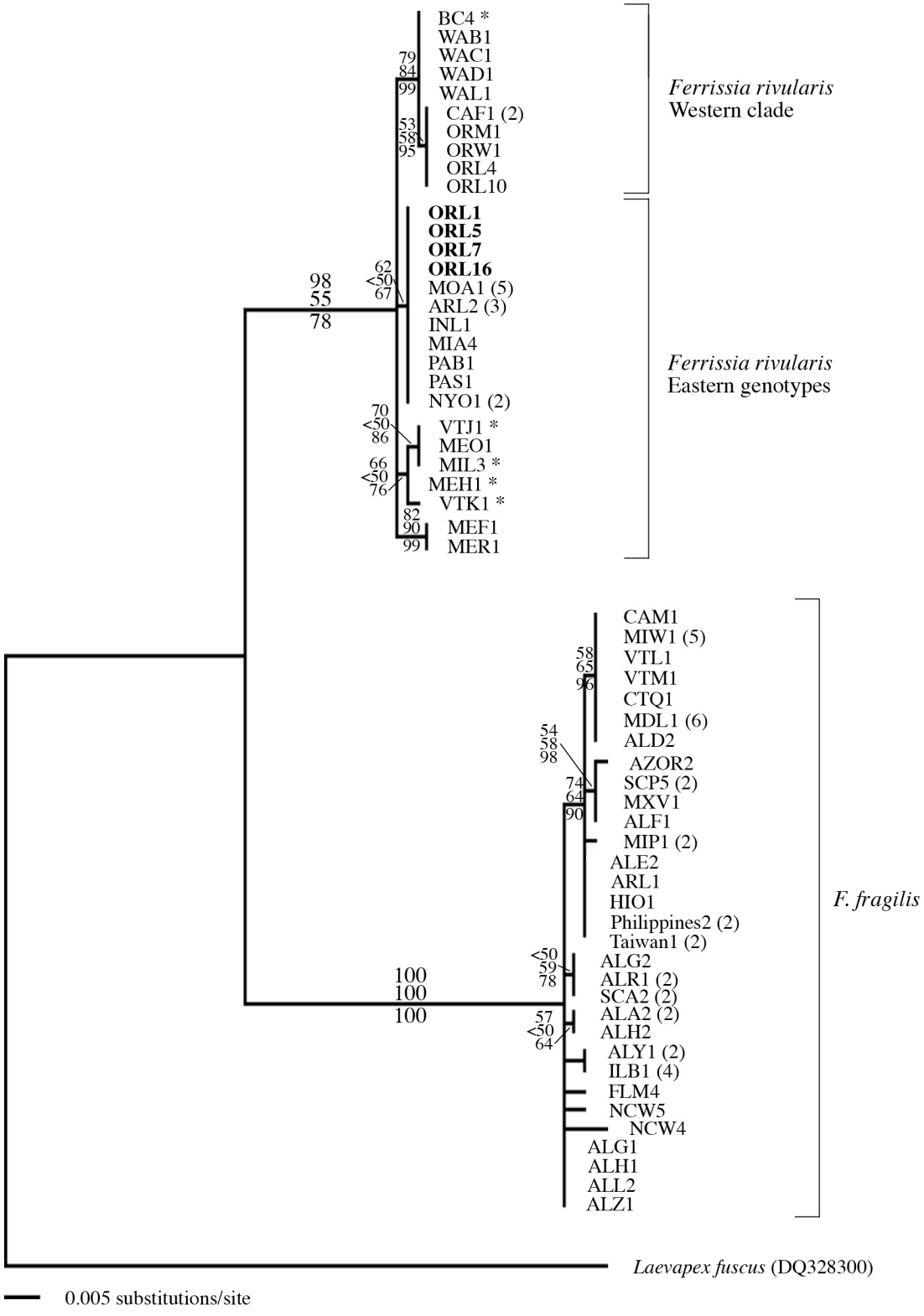
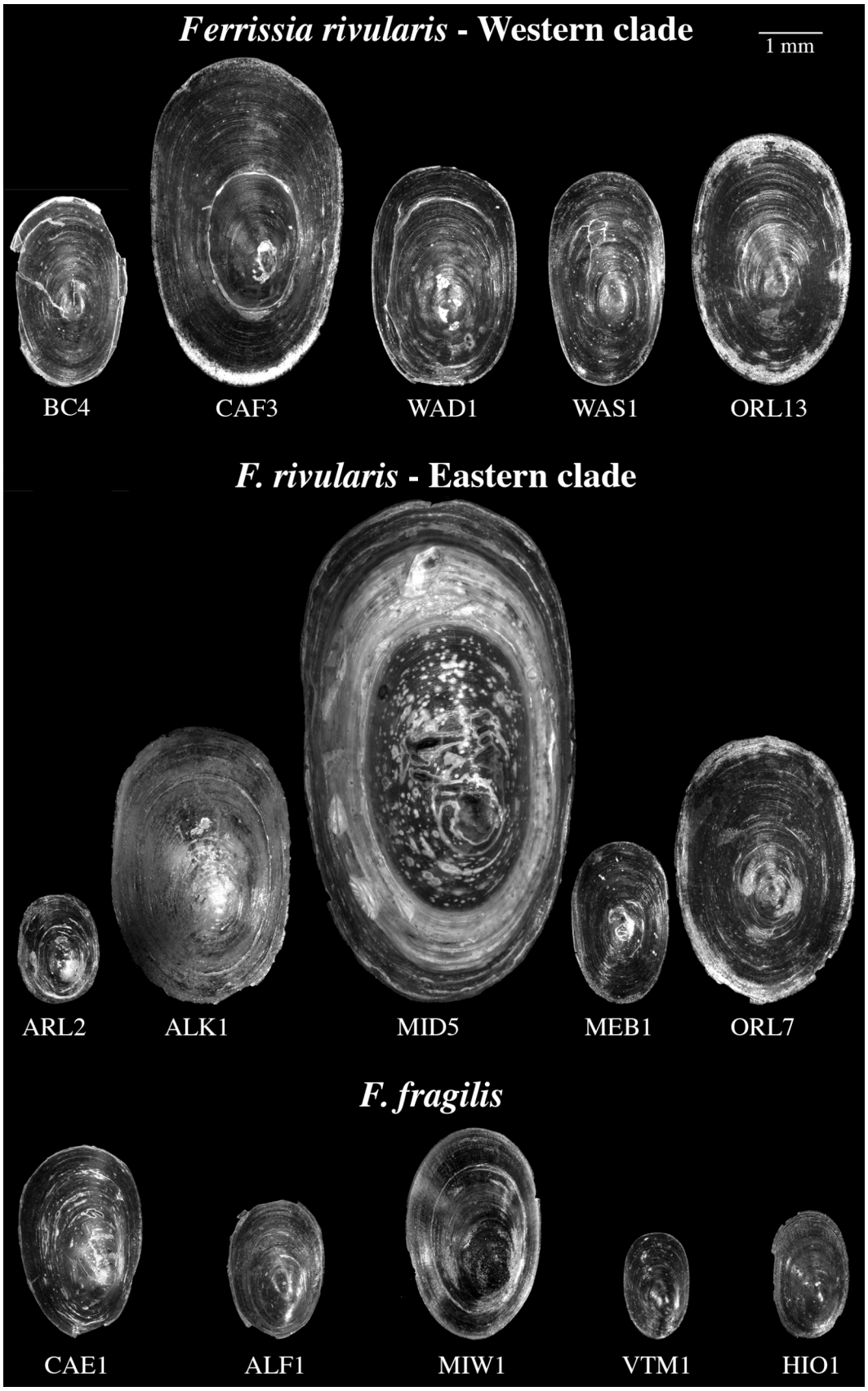

BC4 *
WAB1
WAC1
WAD1
WAL1
CAF1 (2)
ORM1
ORW1
ORL4
ORL10
Ferrissia rivularis
Western clade
ORL1
ORL5
ORL7
ORL16
MOA1 (5)
ARL2 (3)
INL1
MIA4
PAB1
PAS1
NYO1 (2)
VTJ1 *
MEO1
MIL3 *
MEH1 *
VTK1 *
MEF1
MER1
Ferrissia rivularis
Eastern genotypes
CAM1
MIW1 (5)
VTL1
VTM1
CTQ1
MDL1 (6)
ALD2
AZOR2
SCP5 (2)
MXV1
ALF1
MIP1 (2)
ALE2
ARL1
HIO1
Philippines2 (2)
Taiwan1 (2)
ALG2
ALR1 (2)
SCA2 (2)
ALA2 (2)
ALH2
ALY1 (2)
ILB1 (4)
FLM4
NCW5
NCW4
ALG1
ALH1
ALL2
ALZ1
F. fragilis
Laevapex fuscus (DQ328300)
0.005 substitutions/site

Figure 5.6. Photographs of shells vouchers from representatives of the two major clades of *Ferrissia*: *F. rivularis* (eastern and western clades) and *F. fragilis*. It should be noted that individuals BC4, MID5, and MEB1 were originally identified as *F. parallela* based on Table 5.1, though they emerged in my nuclear and mitochondrial gene trees as *F. rivularis*. Additionally, individual ALF1 was identified as *F. mcneilli*, MIW1 was identified as *F. walkeri*, and HIO1 was identified as *F. sharpi* based on sampling localities. All three emerged as *F. fragilis* in representative gene trees.


Ferrissia rivularis - Western clade
1 mm
BC4
CAF3
WAD1
WAS1
ORL13
F. rivularis - Eastern clade
ARL2
ALK1
MID5
MEB1
ORL7
F. fragilis
CAE1
ALF1
MIW1
VTM1
HIO1

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Chapter VI

Conclusion

In the preceding chapters I seek to address three primary questions that would clear up many of the long-standing systematic uncertainties associated with an obscure, yet ecologically significant group of animals – freshwater limpets in the family Ancylidae. These general questions include 1) whether the family is monophyletic and how it is related to other freshwater basommatophoran lineages, 2) how many of the nominal North American ancylid species are phylogenetically valid, and 3) how taxa within the family are phylogenetically interrelated. I describe how I collected representative samples from throughout the ranges of the nominal species and utilized some of the latest methodologies in molecular evolution to offer insight into the taxonomy and evolutionary history of this group. Along the way, I ended up completing a couple of short studies involving both local and long-distance passive transport of these minute snails by various agents of dispersal.

Chapter I provides a broad overview of the family Ancylidae, ultimately focusing on the North American taxa recognized today. I mention Basch's valuable contributions in assessing North American ancylid systematics (1963), though I argue that his work is decades-old and his methods somewhat outdated and insufficient, which has resulted in persistent taxonomic confusion. With inconsistencies pervading the ancylid literature

and the impending extirpation/extinction of more freshwater limpet species, the need for a better understanding of ancylid systematics is evident.

Throughout the results presented in the subsequent chapters, I offer continued support for a monophyletic Ancylidae nested within the Planorbidae. I also provide molecular evidence for two distinct ancylid clades, corresponding to the subfamilies Laevapecinae (*Laevapex*, *Hebetancylus*, *Gundlachia*, *Uncancylus*) and Ancylinae (*Ferrissia*, *Rhodacmea*, *Ancylus*). Unfortunately, while my work incorporates more ancylid taxa than any other molecular phylogenetic study to date, many of the critical nodes that would otherwise reveal sister relationships among the ancylid genera are not robust. Thus, I cannot make definitive statements about intergeneric relationships within the Ancylidae beyond confidently dividing the constituent genera between the two subfamilies.

In my work on *Laevapex* (Chapter II), nuclear markers revealed only a single lineage, corresponding to the type species *L. fuscus*. I found no evidence for the nominal *L. diaphanus*, *L. peninsulae*, or any other *Laevapex* species in synonymy, a result that was corroborated with a geometric morphometric study I completed on the *Laevapex* shell vouchers. My mt COI gene tree did reveal some hidden haplotype diversity as 6 of 109 individuals sequenced for COI emerged in a single divergent lineage of *Laevapex*, though because this result is unsubstantiated by nuclear markers, individuals in this divergent clade cannot be raised to species status. Instead, it is presumed that the divergent haplotypes stem from either introgression or persistent ancestral polymorphisms. Having

a single polymorphic species of *Laevapex* does make identifying members of this genus easier. It just needs to be understood that subtle shell shape differences among populations of *Laevapex* oftentimes represent ecophenotypic variation and not underlying genetic differences. This is not to say without doubt that only one species of *Laevapex* occurs in North America, but based on the samples I studied, there is currently no evidence for other species.

Adding more of an ecological spin to my *Laevapex* study, for Chapter III I was fortunate to document a recent case in which several *L. fuscus* individuals were found attached to the hemelytra of *Belostoma flumineum*, suggesting that these insects serve as an agent of local dispersal for freshwater limpets. Until this discovery, ancylids had only been found on beetles. The impact of such instances of local dispersal on the broader range of ancylids has yet to be quantified, though theoretically over time, the impact could be significant.

In Chapter IV, I provide evidence for the long-distance passive dispersal of ancylids when I discuss how I found that the North American *Ferrissia fragilis* has cryptically invaded Europe and Asia. Two nearly simultaneous discoveries led me to this conclusion: 1) an erroneous finding published by Jørgensen et al. (2004) in which a *F. fragilis* found in Denmark was mistaken for the native *Acroloxus lacustris*, and 2) obtaining *F. fragilis* sequences from Taiwanese and Philippine samples originally identified as *Pettancylus* sp. More recent *Ferrissia* acquisitions from France and the Azores offer evidence of *F. fragilis* having invaded these countries as well. I also show

in Chapter IV that the South American ancylid *Uncancylus* sp. has invaded the Philippines and surmise that the intercontinental transport of ancylids occurs via passive dispersal, perhaps through the aquarium trade. These findings have profound ecological implications, as they exemplify how freshwater ecosystems are being reshaped by ongoing human-mediated transoceanic exchange, a growing threat to native fauna in every region of the world.

My phylogenetic study of *Ferrissia*, presented in Chapter V, revealed that two major *Ferrissia* lineages occur in North America, corresponding to the type species *F. rivularis* and the global invasive *F. fragilis*. There is no molecular evidence for *F. parallela*, *F. walkeri*, *F. mcneilli*, the Hawaiian *F. sharpi*, or any other *Ferrissia* taxa in North America, though rare divergent COI haplotypes are present in some southeastern populations. This should make identifying *Ferrissia* easier, though distinguishing between small specimens of *F. rivularis* and *F. fragilis* is still difficult. Interestingly, there is no unambiguous support for a monophyletic *Ferrissia*, and should *F. fragilis* be more closely related to *Ancylus* and *Rhodacmea*, it is possible that *F. fragilis* will eventually be placed in a different genus. Furthermore, the *F. rivularis* clade reveals geographic structure between eastern and western samples. Oregonian individuals emerging among the eastern nuclear ITS-2 and mt COI sequences indicates the movement of individuals from east to west without subsequent introgression, thus speciation may be occurring.

My overall goal to resolve some of the long-standing systematic discrepancies and

ambiguities complicating North American ancylid studies has been achieved, though, given the expansive range of these animals, further sampling and molecular work would offer continued insights. Conclusions I have drawn here are based solely on the specimens that I had available, and there could easily be ancylid diversity that I did not sample. Nonetheless, my molecular findings have detected hidden genetic diversity in ancylids, that not visible morphologically, and they have also revealed where perceived morphological diversity has no genetic basis and therefore does not warrant separate species designations. By presenting my suggested systematic revision, conservation efforts may now be focused on those North American ancylid species that are truly imperiled.

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