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Identification of 'Extinct' Freshwater Mussel Species Using DNA Barcoding

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Keywords
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Comments
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DNA BARCODING

Identification of 'extinct' freshwater mussel species using DNA barcoding

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Abstract

Freshwater mollusks are highly imperiled, with 70% of the North American species extinct, endangered, or at risk of extinction. Impoundments and other human impacts on the Coosa River of Alabama, Georgia and Tennessee of the southeastern USA alone are believed to have caused 50 mollusk species extinctions, but uncertainty over boundaries among several putatively closely related species makes this number preliminary. Our examination of freshwater mussels collected during an extensive survey of the upper-drainage basin, DNA barcoding and molecular phylogenetic analyses confirm the rediscovery of four morpho-species in the genus *Pleurobema* (Unionidae) previously thought to be extinct from the upper Coosa basin. A fifth 'extinct' form was found in an adjoining basin. Molecular data show that the Coosa morphologies represent at least three species-level taxa: *Pleurobema decisum, P. hanleyianum* and *P. stabile*. Endemism is higher than currently recognized, both at the species level and for multispecies clades. Prompt conservation efforts may preserve some of these taxa and their ecosystem.

Keywords: *cox1*, endangered species, molecular barcode, *Pleurobema*, Unionidae

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Introduction

Nonmarine mollusks such as unionid mussels have disproportionately high rates of extinction and imperilment, but receive little conservation management compared with charismatic vertebrate species (Lydeard *et al*. 2004). Unionids have attracted many researchers because of their ecological significance, economic importance (chiefly in the cultured pearl industry), local abundance, complex life cycle including an obligate parasitic larva, and recent drastic decline (Strayer *et al*. 2004). Like other freshwater organisms such as fishes (Walsh *et al*. 1995), snails (Bogan *et al*. 1995) and crayfishes (Crandall & Templeton 1999), unionid mussels show exceptional diversity and endemism in the southeastern USA (Williams & Neves 1995), where their varied forms have inspired colourful common names such as warrior pigtoe, painted clubshell, inflated heelsplitter, pistolgrip and spike.

The upper Coosa basin extends through Tennessee, Georgia and Alabama in the USA, has an area of approximately 6400 km^2, and drains four physiographic provinces. The watershed has a complex and ancient geological history, dating back at least to the Cretaceous if not to the Palaeozoic, with stream capture and sea level changes producing varying connection and isolation relative to nearby drainages (Adams 1929; Conant 1964; Rindsberg 2003). Although currently part of the Mobile River system, sea level variation in the geological past has isolated the Coosa from other major rivers in the system (Fig. 1).

Historically, the upper Coosa was home to over 40 species of freshwater mussels, making it one of the most biologically

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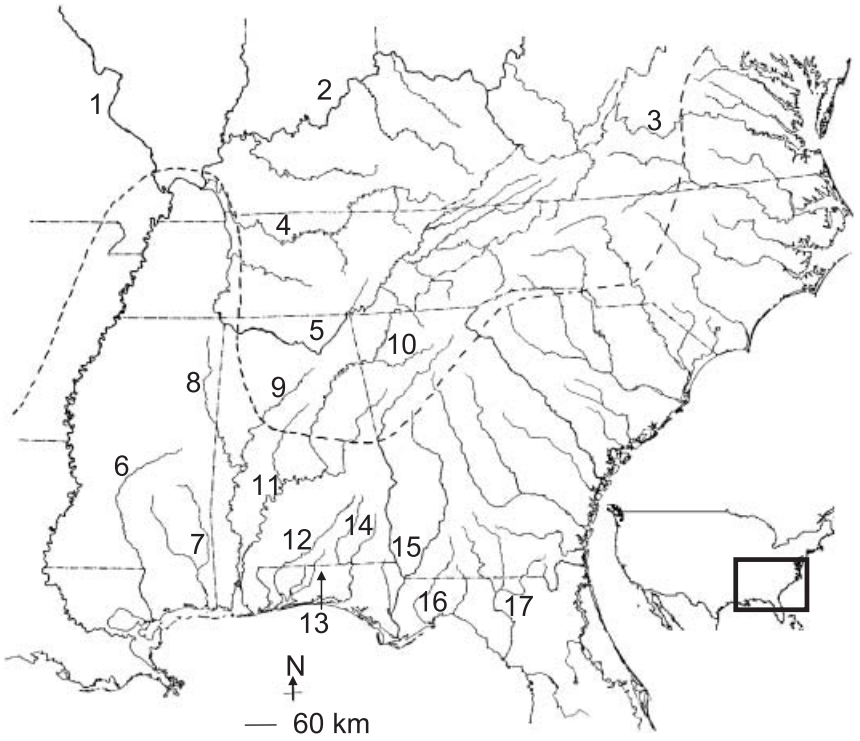

Fig. 1 Biogeographical patterns in *Pleurobema* in the eastern USA. Major river systems are numbered. Mississippi, Ohio, and upper Apalachicola largely coincide with state boundaries. Tennessee (5) -Cumberland (4) -Ohio (2) -Mississippi (1) [*P. clava*, *P. cordatum*, *P. gibberum* (Cumberland only), *P. oviforme* (Tennessee and Cumberland only), *P. rubrum*, *P. sintoxia*]; Pearl (6) and Pascagoula (7) (*P. beadlianum*); Tombigbee (8) - Alabama (11) (*P. perovatum*, *P. taitianum*; *P. decisum*); Black Warrior (9) (*P. furvum, P. rubellum*); Escambia (12), Yellow (13), and Choctawhatchee (14) (*P. strodeanum*); Coosa (10) (*P. chattanoogaense*, *P. decisum*, *P. hanleyianum*, *P. stabile*, *P. troschelianum*); Apalachicola (15), Ochlockonee (16), and Suwanee (17) (*P. pyriforme*); James (3) (*P. collina*). Dashed line indicates approximate Oligocene highstand shoreline, showing past isolation of major rivers (new data).

diverse rivers in the world (Garner *et al*. 2004). The decline of the mussel fauna began in the early 1900s in response to the building of locks and dams and later to point-source pollution from textile and carpet-dying operations, nonpoint pollution from urban and suburban sprawl, and siltation from poor land-use practices (Mirarchi *et al*. 2004). The destruction and alteration of stream and floodplain habitat and reduction of water quality resulted in a catastrophic decline of freshwater mollusks. Most species suffered drastic range reductions, with about 50 mollusk species entirely eliminated. This includes all species of three snail genera (*Gyrotoma*, *Amphigyra* and *Neoplanorbis*) and severe reduction of two other snail genera that were thought to be extinct, but were later rediscovered (*Tulotoma* and *Clappia*) (Hershler *et al*. 1990; Garner *et al*. 2004; S. A. Clark personal communication). Among unionid bivalves, the subgenus *Alasmidens* is presumed extinct (Clarke 1981). *Pleurobema* lost nine species in the Coosa. *Pleurobema* is one of the most speciose genera of freshwater mussel but also one of the most imperiled. The most recent comprehensive summary tentatively recognized 32 species (the majority from the Mobile River basin), of which 13 (40%) were thought to be extinct (Turgeon *et al*. 1998) and therefore have no official protected status. Twelve purportedly extant *Pleurobema* species are federally listed as Endangered in the USA (Turgeon *et al*. 1998). The only bivalve genus with more recently extinct species is *Epioblasma*, most species of which occurred in the Tennessee, Cumberland and Ohio systems.

Complicating efforts to assess conservation status in freshwater mussels is the difficulty in identifying specimens to species for taxonomically challenging taxa (Roe 2000) including *Pleurobema*, whose shells often differ only by subtle characteristics. Furthermore, most species and genera are currently defined using historical morphological concepts. Although many have been included in molecular phylogenetic studies, only a few genera have been thoroughly re-investigated phylogenetically. Recent molecular analysis of the 45 currently recognized North American genera in Ambleminae revealed that most polytypic genera are polyphyletic (Campbell *et al*. 2005), highlighting the problems in current classification. In particular, this means that a species-level study cannot assume that close relatives are currently assigned to the same genus. Among unionids, identifying and delimiting species within *Pleurobema* based on shell morphology is especially problematic (Goodrich 1913; Simpson 1914; Burch 1975; Turgeon *et al*. 1998). Shell shape in unionids reflects many environmental parameters, potentially over decades of growth. *Pleurobema* generally lacks significant shell sculpture or other distinguishing features (the exception, *Pleurobema collina*, usually has spines, but the present data indicate it is not a true *Pleurobema*), so species are currently identified by subjective assessment of shell shape. Soft-part anatomy is poorly documented, and anatomical differences between closely related species, when known, are often subtle, requiring detailed examination.

In such situations, molecular techniques such as DNA barcoding have great potential to supplement traditional taxonomic methods. Many recent studies have successfully applied these techniques to other animals (Hajibabaei *et al*. 2006; Kelly *et al*. 2007; Kerr *et al*. 2007). The *cox1* gene has been widely used in studies on freshwater mussels over the past decade (Hoeh *et al*. 1997; Roe & Hoeh 2003; Araujo *et al*. 2005; Campbell *et al*. 2005; Gustafson & Iwamoto 2005; Källersjö *et al*. 2005; Soroka 2005; Graf & Cummings 2006; Walker *et al*. 2006; Zanatta & Murphy 2006, references therein), so a good comparative data set is available. However, many species remain undocumented, limiting the potential for barcode-type approaches to identification of unknowns. Jones *et al*. (2006) had difficulty distinguishing some mussel species using other mitochondrial genes and ITS1, but microsatellites showed clear differences. Nevertheless, most studies on unionids have found mitochondrial genes to be very useful. Potential pitfalls for barcoding have also been documented for other taxa and for theoretical models of speciation (Hickerson *et al*. 2006; Meier *et al*. 2006). These highlight the importance of investigating additional molecular, morphological and other data in addition to the barcode sequence. Although doubly uniparental inheritance of mitochondrial DNA produces some problems for other bivalves, in unionids the male mitotype is strictly associated with the male germ line, so that sampling of somatic tissue yields only female mitotypes. Also, there is no evidence of exchange between the male and female mitotypes within Unioniformes, and the male mitotypes are so divergent from the female as to be readily recognizable (Walker *et al*. 2006).

We sought to determine the level of molecular differentiation between morphological forms in *Pleurobema* species from the upper Coosa system. In turn, we used these data to identify molecular markers suitable for identification of problematic specimens and to place the species into a phylogenetic framework. Additionally, phylogenetic analyses that incorporate the actual sequence data provide a more sensitive test of patterns of molecular differentiation than simply comparing percentage differences. For molecular data, species differentiation was based on monophyly (i.e. a phylogenetic species concept) and the per cent difference (i.e. a phenetic criterion widely used for barcoding studies).

Large sample sizes are desirable to test the level of intrapopulation variability; however, in some cases our sample of one specimen was the entire population. Both the extreme rarity of most species and endangered species regulations limited the number of modern samples available. Within the Mobile basin, a few healthy populations are known only for *P. decisum* and *P. perovatum*, both of which are listed as Endangered.

Materials and methods

In 1998, an intensive programme was initiated to survey the upper Coosa River basin with an emphasis on the historically richest sub-basin, that of the Conasauga River. To date, over 700 sites in the upper Coosa River system have been surveyed for mussels and other invertebrates. Annual surveys of the Conasauga River began in 1998, but heavy rain prevented the 2002 survey. Similar surveys have examined other areas in the Mobile basin, emphasizing the few relatively undisturbed portions of larger rivers. Depending on water depth, surveying required wading, snorkeling and/or scuba diving to search for mussels in the river bed. In 1998–1999, 616 mussel specimens were found in the upper Coosa basin representing 24 species, in 2002–2003, 345 mussels were found representing 18 species, and in 2005–2006, 565 mussels of 20 species were found, for a total of 28 species, including two impoundment-tolerant species not recorded historically from the upper Coosa.

Current species taxonomy is based on shell shape, colour pattern and geographical distribution. In particular, *Pleurobema* species differ in degree of elongation and whether they are more oval, quadrate, or triangular. Comparison of our often eroded specimens to museum material helped verify their identity, especially when large suites, illustrating intrapopulation variation, were available. Colour pattern is somewhat variable and often obscured in older specimens, in addition to the influence of erosion and encrustation, but may be helpful if it is visible. For example, *Pleurobema chattanoogaense* typically has a few green spots on the early part of the shell (visible near the dorsal margin in Fig. 2) giving it the common name of painted clubshell, whereas *Pleurobema stabile* is all brown.

To determine the taxonomic identity of unknown *Pleurobema* specimens, we used molecular phylogenetic methods to construct topologies of relatedness between morphologically identified species and unknown specimens. DNA was extracted from fresh, frozen, or ethanol-preserved specimens using standard cetyltrimethyl ammonium bromide (CTAB) and chloroform–isoamyl alcohol protocols (Winnepenninckx *et al*. 1993). Voucher specimens for all new sequences are in the University of Alabama collections except for *Pleurobema pyriforme*, in the North Carolina Museum of Natural History. All but two *Pleurobema* species with known extant populations were sequenced. We could not obtain sequences for *Pleurobema plenum*, an endangered species from the Tennessee and Ohio river systems closely related to *Pleurobema cordatum*, *P. rubrum* and *P. sintoxia*, nor specimens for *P. riddelli* from west Louisiana and east Texas. Taxa representing other genera of the tribe Pleurobemini, including all extant species from the Coosa, and other tribes of the subfamily Ambleminae served as outgroups.

We selected two mitochondrial genes that had worked well in previous studies on unionids, cytochrome oxidase

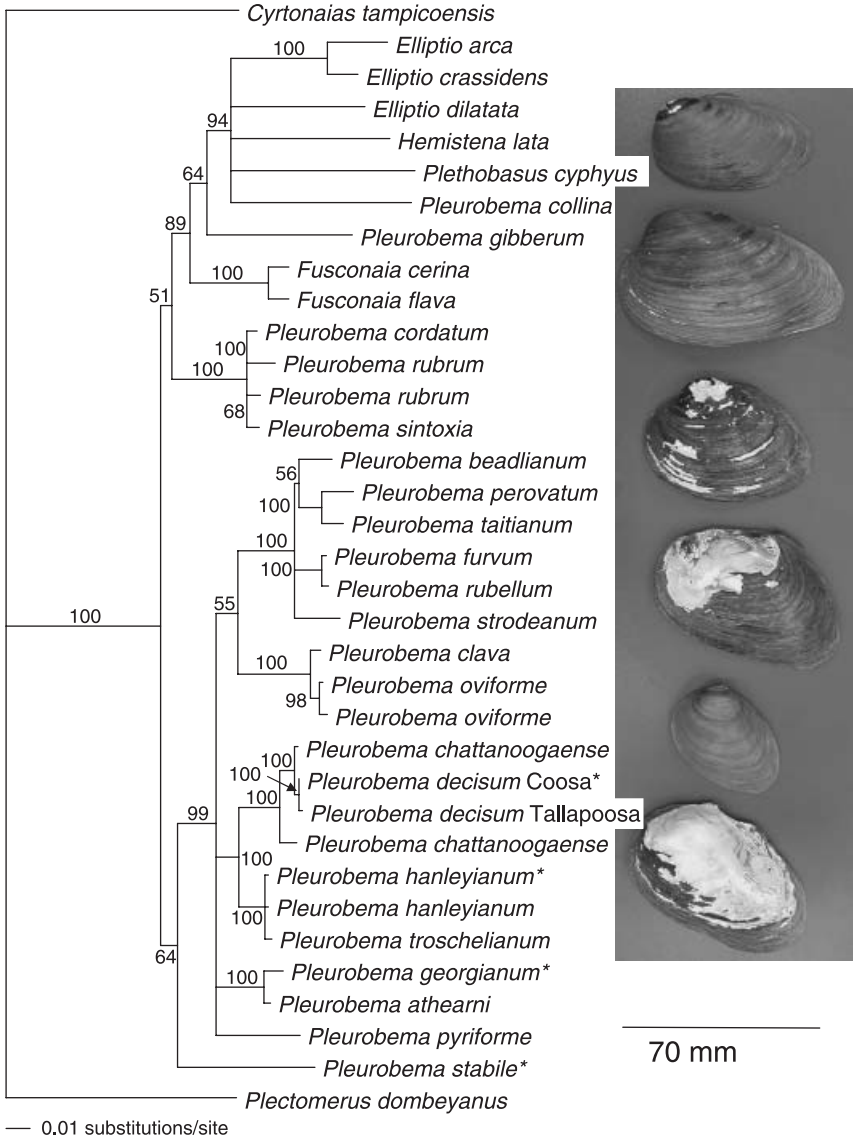

Fig. 2 Phylogram showing Bayesian analysis on *cox1* and *nadh1* sequence data. Results of the parsimony analyses were similar (Fig. 6). Burn-in was 10 000, mean ln likelihood was –8252.920. Numbers are posterior probabilities. The branch uniting *Pleurobema cordatum* and *Pleurobema rubrum* and that uniting *P. rubrum* and *P. sintoxia* are too short to be visible, despite having 100% and 68% probability, respectively. Asterisks indicate figured specimens. The shells are left valves of several upper Coosa *Pleurobema* species from University of Alabama collections. From the top: *Pleurobema chattanoogaense* (historical specimen), *P. decisum*, *P. hanleyianum*, *P. georgianum*, *P. stabile* (collected 1912), *P. stabile* (specimen collected 2001). The new *P. stabile* specimen is 70 mm in maximum dimension. Despite the heavy erosion in the second specimen, the posterioventral elongation in both specimens of *P. stabile* distinguishes them from the other species.

I (*cox1*) and NADH dehydrogenase subunit 1 (*nadh1*), and one nuclear region used in a few prior studies, the ribosomal internal transcribed spacer I (ITS1). Primers for *cox1* were 5′-GTTCCACAAATCATAAGGATATTGG-3′ and 5′-TACACCTCAGGGTGACCAAAAAACCA-3′, adapted from Folmer *et al*. (1994), primers for *nadh1* were 5′-TGGCAGAAAAGTGCATCAGATTTAAGC-3′ and 5′-GCTATTAGTAGGTCGTATCG-3′ (Buhay *et al*. 2002; Serb & Lydeard 2003), and primers for ITS1 were 5′-AAAAAGCTTCCGTAGGTGAACCTGCG-3′ and 5′-AGCTTGCTGCGTTCTTCATCG-3′ (King *et al*. 1999). Polymerase chain reaction (PCR) cycles were: 92 °C 2 min; 92 °C 40 s 40 °C 40 s 72 °C 90 s 5×; 92 °C 40 s 50 °C 40 s 72 °C 90 s 25×; 72 °C 10 min; hold 4 °C. PCR products were purified using QIAquick PCR purification kits. Cycle sequencing used ABI BigDye Terminator kits with thermal cycle parameters of 1 °C per second ramp speed, starting with 1 min at 96 °C followed by 26 cycles of 96 °C for 10 s, 49 °C for 5 s, and 60 °C for 4 min, then 10 min at 60 °C and hold at 4 °C. The cycle sequencing products were purified with sephadex columns or QIAGEN DyeEx kits and then run on an automated sequencer (ABI 3100). The results for each strand were compared and aligned with published sequences using BIOEDIT (Hall 1999). No indels were found in the protein-coding genes, but ITS1 has several. New *cox1* sequences have been identified as barcode data in GenBank. Although ITS1 can show significant variation within individuals, all included specimens yielded sequences that were readily readable without cloning. This indicates that only one copy of the gene was amplifying, as found in some other studies on unionids (Grobler *et al*. 2005; Jones *et al*. 2006). Several other unionids have also yielded either a single sequence or else two alleles differing by a single base in a repeat region, whereas almost all gastropods we

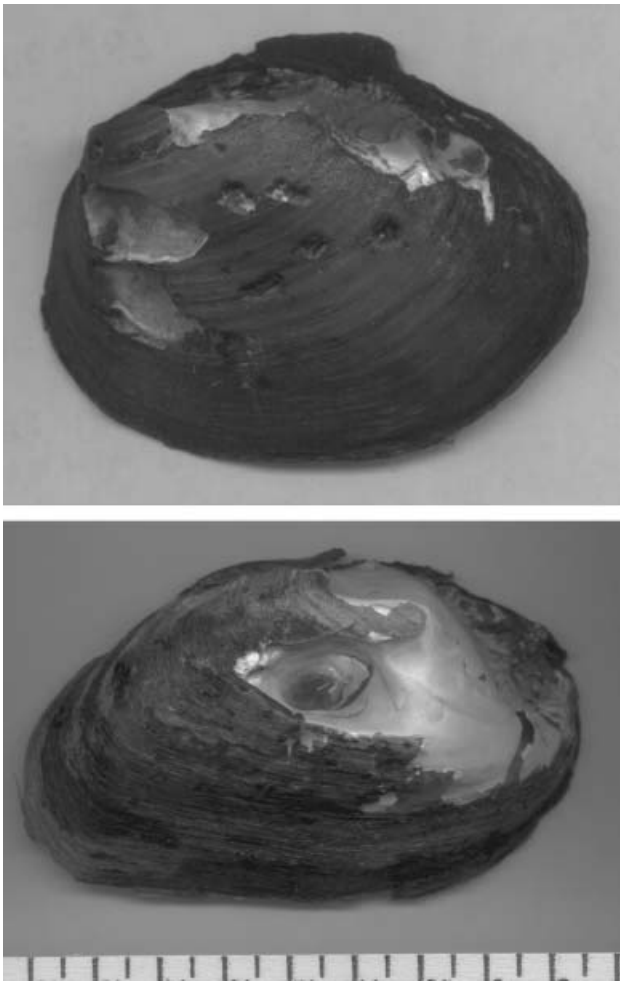

Fig. 3 Aberrant specimens of *Pleurobema decisum* from the Tallapoosa system (UAUC3299, top) and from the Coosa system (UAUC471, bottom), identified based on *nadh1* sequence. Contrast with the normal specimen in Fig. 2.

have tried yielded multiple divergent sequences, unreadable without cloning (personal observation).

Phylogenetic analyses of the sequences included heuristic parsimony searches and bootstrap analyses in PAUP*4.10 (Swofford 1998). Because *cox1* and *nadh1* yielded similar results, a partition-homogeneity test was run in PAUP* (P_{ILD} of Dowton & Austin 2002) with 1000 replicates of 10 random addition replicates each. The maximum number of trees per replicate was set to 10 000. This test is sensitive to other factors, such as partition size and evolutionary model, besides data compatibility (Dowton & Austin 2002), but may provide a rough idea of agreement between data sets. Despite the problems of the ILD type of tests, no better alternative has gained wide acceptance. The *P* value was 0.65, so the two mitochondrial genes were concatenated for further analysis. Bayesian analysis, using MRBAYES 3.1.1 (Ronquist & Huelsenbeck 2003), served as an alternative phylogenetic method. Bootstrap values typically underestimate support, whereas Bayesian probabilities tend to overestimate it (Simmons *et al*. 2004). Maximum parsimony analyses used 1000 random replicates, hold = 10, swap = TBR. Bootstrap analyses used 1000 replicates, each using a random parsimony search of 10 replicates. For the ITS1 data, parsimony and bootstrap analyses treated gaps as a fifth base. Bayesian analysis used 2 000 000 generations and eight chains; revmat, shape, pinvar and statefreq were unlinked. MRMODELTEST 2.2 (Nylander 2004) recommended K80+g for ITS1 and GTR+I+G independently selected for both *nadh1* and *cox1*. In the Bayesian analyses, the standard deviation of split frequencies went under 0.01 for both. All bootstrap percentages and Bayesian probabilities over 50% for branches in the maximum parsimony trees are shown. Some analyses had 55% or less bootstrap support for a clade not in the strict consensus; these are not indicated. The sequences used in this study are listed in Table 1.

As we accumulated *cox1* and *nadh1* sequence data, all but one specimen showed close correspondence to sequences from positively identified specimens. That badly eroded specimen (Fig. 2) had been tentatively assigned to *P. chattanoogaense*, but based on the molecular data, it was highly distinct from all other sampled specimens. The anomalous molecular results prompted further morphological study of museum specimens, along with re-examination of the mystery specimen, to identify morphological characters that were not obliterated by the erosion.

To determine the geological history of drainage systems, we examined stream drainage patterns, erosional features, sediment outcrop areas and other geomorphological features as well as literature data. In turn, the drainage histories were compared to the biogeographical patterns seen in the phylogenies.

Results

The intensive searches in the upper Coosa yielded live or freshly dead specimens still suitable for molecular genetic analysis from four supposedly extinct morphospecies of *Pleurobema*: painted clubshell, *P. chattanoogaense* (Lea 1858); Georgia pigtoe, *P. hanleyianum* (Lea 1852); Alabama clubshell, *P. troschelianum* (Lea 1852) and one badly eroded individual that, after molecular analyses and detailed analysis of museum specimens, was identified as *Pleurobema stabile* (Lea 1861) [often listed under the junior synonym *Pleurobema murrayense* (Lea 1868)] (Fig. 2). Another very eroded specimen resembles *Pleurobema hartmanianum* (Lea 1860), but it has not yet yielded DNA sequences. Other unusual specimens were assignable to recognized species based on DNA sequence data (Fig. 3). Molecular data also confirmed that all of the *Pleurobema perovatum*-like specimens found in the upper Coosa were in fact *P. hanleyianum*.

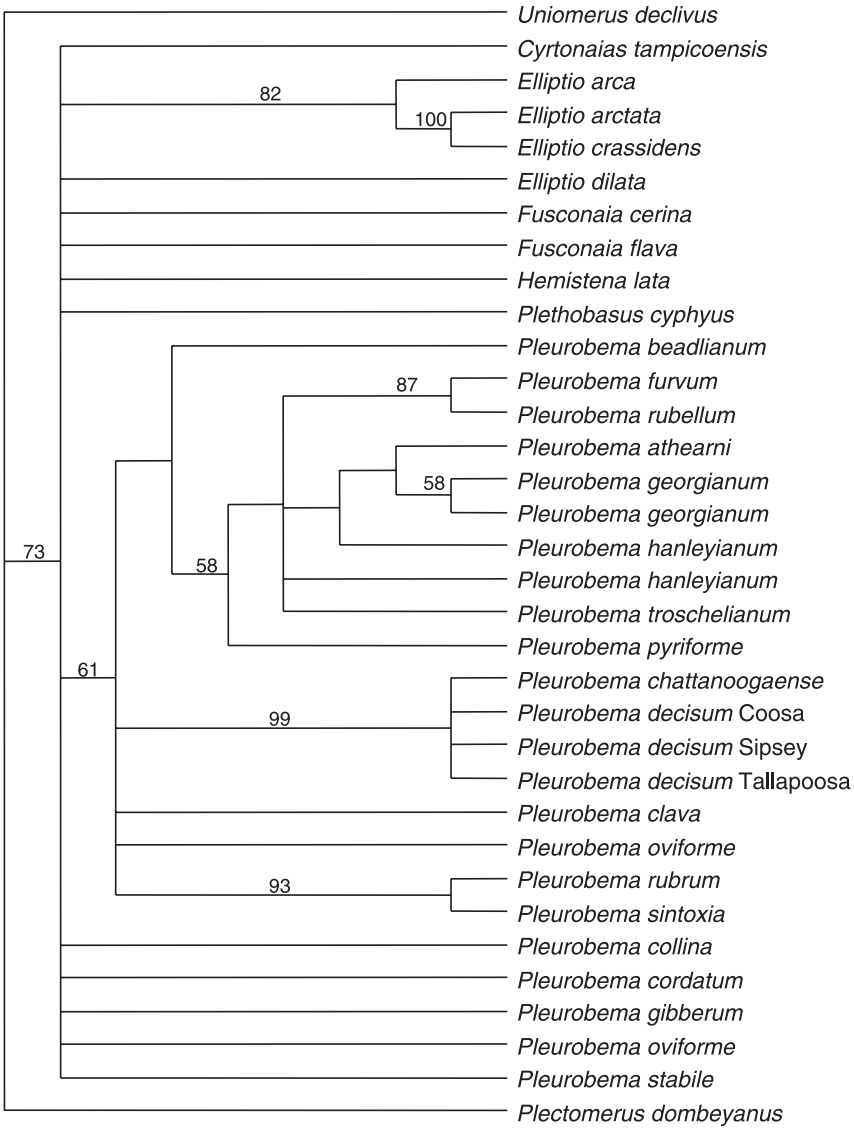

Fig. 4 Strict consensus of 861 maximum parsimony trees, length 492, ITS1 data. Numbers indicate bootstrap percentage if over 50%.

Some of these species have not been reported as alive for decades (Evans 2001). *Pleurobema stabile* was last collected and reliably identified in 1958 by H. Athearn (Museum of Fluviatile Mollusks collections, P.D.J., personal observation). Additional sampling in other parts of the Mobile basin yielded the warrior pigtoe, *Pleurobema rubellum*, in the upper Black Warrior River system. This species has historically been reported from the upper Coosa and currently is listed as Extinct (Turgeon *et al*. 1998). However, no specimens of the western Mobile basin species *Pleurobema curtum* or *Pleurobema marshalli* were found, despite their current listing as Endangered rather than Extinct. Live specimens of these have not been found since the 1980s.

Both mitochondrial genes yielded similar results, showing several well-supported clades within *Pleurobema* (Figs 2 and 6). Intraspecies variation is low. The results from ITS1 are generally less well-resolved and less well-supported than from the other two genes (Figs 4 and 5), with more intraspecies variation, but it provides evidence for the distinctiveness of some species. *Pleurobema* does not appear to be monophyletic. When *cox1* or *nadh1* were analysed separately, including sequences from specimens that amplified for only one gene, all sequences for a species placed in the same clade, and those clades had at least 89% bootstrap support (not shown). Table 2 shows the per cent difference between various taxa for each gene region.

Discussion

The tribe Pleurobemini includes approximately 90 species in the genera *Elliptio*, *Fusconaia*, *Hemistena*, *Lexingtonia*, *Plethobasus*, *Pleurobema* and *Quincuncina* (Turgeon *et al*. 1998; Campbell *et al*. 2005). Molecular analyses indicate that the current generic classification of freshwater mussels

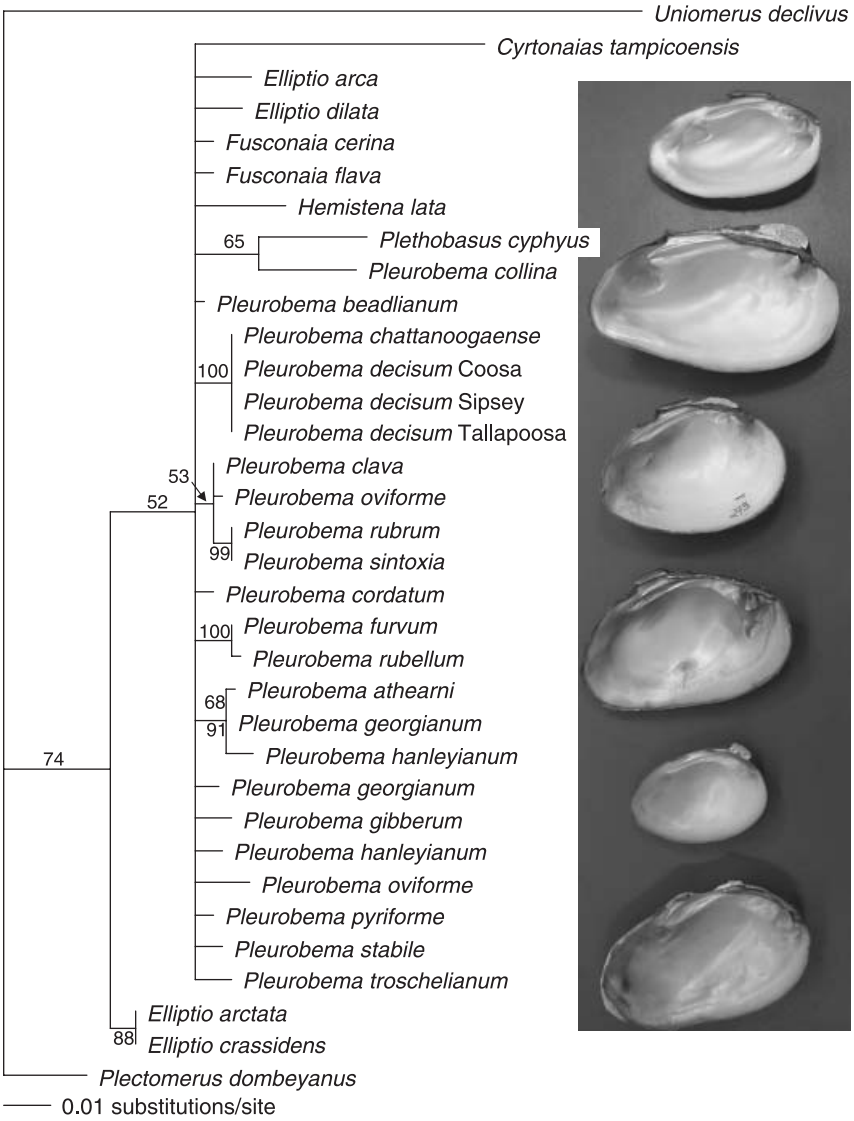

Fig. 5 Phylogram showing Bayesian analysis on ITS1 sequence data. Burn-in was 30800, mean ln likelihood was –1978.885. Numbers are posterior probabilities. The branch uniting *Pleurobema athearni* and *P. georgianum* is too short to be visible, despite having 68% probability. Interiors of left valves of specimens from Fig. 2 shown. From the top: *Pleurobema chattanoogaense*, *P. decisum*, *P. hanleyianum*, *P. georgianum*, *P. stabile* (museum specimen, collected 1912), *P. stabile* (specimen collected 2001). The new *Pleurobema stabile* specimen is 70 mm in maximum dimension. The different position of the posterior (left) adductor muscle scar in *P. stabile* vs. *P. chattanoogaense* shows a different body configuration in the shell despite having similarly ovate outlines.

requires extensive revision, and some species are even assigned to the wrong tribe (Roe & Lydeard 1998; King *et al*. 1999; Buhay *et al*. 2002; Serb *et al*. 2003; Campbell *et al*. 2005). Most extant species currently assigned to *Pleurobema*, including its type species, *Pleurobema clava* (Lamarck 1819), comprise a clade, thus largely but not entirely supporting current taxonomy.

For the mitochondrial genes, both phenetic distance and phylogenetic placement generally did well at sorting out morphologically distinct species. Kandl *et al*. (2001) likewise was able to separate problematic *Pleurobema* species in the Gulf Coast drainages east and south of the Mobile basin, using a short segment of the *cox1* gene. Their sequences place in the same clades as our sequences for the same species (personal observation), but because they are much shorter they were not included in the present analyses.

Within *Pleurobema*, several smaller clades are largely congruent with major rivers (Figs 1 and 2), reflecting their long independent histories over geological time. Except for *Pleurobema decisum*, most species are confined to a single basin or group of associated river systems. This contrasts with existing groupings based on shell morphology, which range across drainages. For example, *Pleurobema taitianum* from the Tombigbee and Alabama systems resembles the *Pleurobema sintoxia* group from the Tennessee, Ohio, and Mississippi systems in its relatively triangular, heavy shell, and *P. decisum* and *Pleurobema chattanoogaense* from the Coosa resemble the *Pleurobema clava-P. oviforme* group from the Tennessee and Ohio systems in their elongate shape. In turn, the species within a river are typically more closely related to each other than to species from other river systems. Although most rivers have a single clade, and the large Tennessee–Ohio–Mississippi system has two (three if one counts the Cumberland species *Pleurobema gibberum* that belongs in a different genus based on present results), three clades of species occur in the small but ecologically rich

Table 1 Taxa and GenBank accession numbers. Type species of genera are indicated by T. New sequences generated in the present study are indicated by *. Most mitochondrial sequences (the unstarred ones starting with AY) were also generated in this study but previously published in Campbell *et al*. (2005)

Species	*nadh1*	*cox1*	ITS1
Cyrtonaias tampicoensis (Lea 1838) T	AY655090	AF231749	DQ383436*
Elliptio arca (Conrad 1834)	AY655093	AY654995	DQ383437*
Elliptio arctata (Conrad 1834)	no data	DQ383427*	DQ383438*
Elliptio crassidens (Lamarck 1819) T	AY613788	DQ383428*	DQ383439*
Elliptio dilatata (Rafinesque 1820)	DQ385872*	AF231751	DQ383440*
Fusconaia cerina (Conrad 1838)	AY613792	AY613823	DQ383441*
Fusconaia flava (Rafinesque 1820) T	AY613793	AF156510	DQ383442*
Hemistena lata (Rafinesque 1820) T	AY613796	AY613825	DQ383443*
Plectomerus dombeyanus (Valenciennes 1827) T	AY655110	AY655011	DQ383444*
Plethobasus cyphyus (Rafinesque 1820) T	AY613799	AY613828	DQ383445*
Pleurobema athearni (Gangloff *et al*. 2006)	AY655114	AY655015	DQ383446*
Pleurobema beadlianum (Lea 1861)	DQ385873*	DQ383429*	DQ383447*
Pleurobema beadlianum	AY613800*	no data	no data
Pleurobema chattanoogaense (Lea 1858)	AY613801	AY613829	no data
Pleurobema chattanoogaense	AY655111	AY655012	DQ383448*
Pleurobema chattanoogaense	no data	DQ383430*	no data
Pleurobema clava (Lamarck 1819) T	AY613802	AY655013	DQ383449*
Pleurobema clava T	no data	AF231754	no data
Pleurobema collina (Conrad 1837)	AY613803	AY613830	DQ383450*
Pleurobema cordatum (Rafinesque 1820)	AY613804	AY613831	DQ383451*
Pleurobema decisum (Lea 1831) Coosa1	AY613805	AY613832	DQ383452*
Pleurobema decisum Coosa2	DQ383467*	no data	no data
Pleurobema decisum Sipsey1	no data	AF232801	no data
Pleurobema decisum Sipsey2	no data	DQ383431*	DQ383453*
Pleurobema decisum Tallapoosa1	AY655112	AY655014	no data
Pleurobema decisum Tallapoosa2	DQ383466*	no data	DQ383454*
Pleurobema furvum (Conrad 1834)	AY613806	AY613833	DQ383455*
Pleurobema georgianum (Lea 1841)	AY613807	AY613834	DQ383456*
Pleurobema georgianum	AY655113	no data	DQ383457*
Pleurobema gibberum (Lea 1838)	DQ385874*	AY613835	DQ383458*
Pleurobema gibberum	AY613808	no data	no data
Pleurobema hanleyianum (Lea 1852)	AY655115	AY655016	DQ470003*
Pleurobema hanleyianum	AY613809	AY613836	DQ383459*
Pleurobema oviforme (Conrad 1834)	AY613810	AY655017	DQ470004*
Pleurobema oviforme	AY655116	AY613837	DQ383460*
Pleurobema perovatum (Conrad 1834)	AY613811	AY613838	no data
Pleurobema perovatum	no data	DQ383433*	no data
Pleurobema pyriforme (Lea 1857)	AY613812	AY613839	no data
Pleurobema pyriforme	DQ383468*	no data	DQ383461*
Pleurobema rubellum (Conrad 1834)	AY613813	AY613840	DQ383462*
Pleurobema rubrum (Rafinesque 1820)	AY655117	AY655018	no data
Pleurobema rubrum	AY613814	AY613841	DQ470005*
Pleurobema sintoxia (Rafinesque 1820)	AY613815	AY655019	DQ470006*
Pleurobema sintoxia	no data	AF156508	no data
Pleurobema stabile (Lea 1861)	AY613816*	AY613842*	DQ383463*
Pleurobema strodeanum (Wright 1898)	AY613817	AY613843	no data
Pleurobema strodeanum	no data	DQ383434*	no data
Pleurobema taitianum (Lea 1834)	AY613818	AY613844	no data
Pleurobema troschelianum (Lea 1852)	AY613819	AY613845	DQ383464*
Uniomerus declivus (Say 1831)	no data	AY613846	DQ383435*

Table 2 Percentage differences between taxa. *Pleurobema* s.s. excludes *P. collina*, *P. cordatum* group (*P. cordatum*, *P. rubrum*, *P. sintoxia*), *P. gibberum* and *P. stabile*. Between species comparison excludes the possibly conspecific close pairs, separately enumerated (*P. chattanoogaense*–*P. decisum*, *P. clava*-*P. oviforme*, *P. furvum*–*P. rubellum*, *P. georgianum*–*P. athearni*, *P. hanleyianum*–*P. troschelianum*). Numbers given are mean and range of raw percentages (gaps treated as missing data for ITS1). If only a single sequence was available for each species in a comparison, only a single value is given

Comparison	*cox1*	*nadh1*	ITS1
Between genera of Pleurobemini	8.97 (5.39–12.28)	10.18 (7.38–13.85)	1.86 (0.20–4.36)
Between species of *Pleurobema* s.s.	5.63 (1.16–9.08)	5.98 (2.65–9.03)	0.90 (0.19–1.96)
Within species of *Pleurobema* s.s.	1.18 (0.00–2.74)	0.82 (0.13–2.07)	0.42 (0.00–1.17)
Elliptio dilatata–other *Elliptio*	8.12 (7.37–8.51)	11.89 (11.50–12.27)	1.13 (1.01–1.20)
'Pleurobema' collina–other *Pleurobema*	9.27 (8.08–11.27)	11.37 (9.65–13.34)	2.90 (2.39–3.63)
'Pleurobema' cordatum group–other *Pleurobema*	6.90 (3.83–10.04)	8.29 (6.85–11.50)	0.96 (0.39–1.40)
'Pleurobema' gibberum–other *Pleurobema*	9.30 (7.44–11.30)	9.70 (8.70–11.11)	1.40 (0.80–1.97)
'Pleurobema' stabile–other *Pleurobema*	7.07 (5.52–9.30)	10.27 (9.04–12.15)	1.14 (0.79–1.58)
Pleurobema chattanoogaense–P. decisum	1.57 (0.49–2.38)	1.07 (0.26–1.81)	0.00 (0.00–0.00)
Pleurobema clava–P. oviforme	1.48 (0.67–2.22)	1.00 (0.84–1.16)	0.59 (0.20–0.98)
Pleurobema furvum–P. rubellum	0.50	0.39	0.19
Pleurobema georgianum–P. athearni	1.29	1.04 (0.90–1.18)	0.48 (0.19–0.77)
Pleurobema hanleyianum–P. troschelianum	0.64 (0.53–0.75)	0.32 (0.26–0.39)	0.58 (0.20–0.97)

upper Coosa system. *Pleurobema decisum*, *P. chattanoogaense*, *P. hanleyianum* and *P. troschelianum* form one clade of species, all confined to the upper Coosa system except for *P. decisum*, which ranges throughout the Mobile basin. The molecular evidence thus strongly suggests that *P. decisum* originated in the upper Coosa basin and recently spread to other rivers. This idea is also supported by the genetic diversity found in *P. decisum* samples from the Coosa, vs. minimal variation within the populations from other parts of the Mobile basin. A second clade includes only *Pleurobema stabile*, although extinct species such as *P. fibuloides* (Lea 1859) might belong here based on shell features. The third clade present in the upper Coosa includes *Pleurobema georgianum* and the newly described *Pleurobema athearni* (Gangloff *et al*. 2006) from the middle Coosa.

Both the phenetic distances and the phylogenetic results indicate that the upper Coosa forms are distinct from the species endemic to the western Mobile basin. These results contradict earlier morphological studies that suggested that Coosa species might be synonyms of taxa described from other river basins. In particular, *P. hanleyianum* has previously been confused with *Pleurobema perovatum* (Parmalee & Bogan 1998), and *P. stabile* has been synonymized with *Pleurobema rubellum* (Frierson 1927). In contrast, our molecular results indicate that *P. perovatum* and *P. rubellum* are part of a large clade centred on the western Mobile basin, not closely related to Coosa natives.

Some species pairs show minimal molecular divergence (Table 2) and may represent ecophenotypes of a single species or extremely close relatives, including *P. chattanoogaense* and *P. decisum*, *P. hanleyianum* and *P. troschelianum*, *P. furvum* and *P. rubellum*, *P. georgianum* and *P. athearni*, *P. perovatum* and *P. taitianum*, and *P. clava* and *P. oviforme*. However, ITS1 showed greater differences between *P. hanleyianum* and *P. troschelianum* and between *P. clava* and *P. oviforme* than did the mitochondrial genes. ITS1 also showed high intraspecific variation within *P. hanleyianum* and *P. oviforme*, so the significance of the differences for these species is unclear. The species pair *P. georgianum* and *P. athearni* and the pair *P. perovatum* and *P. taitianum* are morphologically quite distinct with mitochondrial per cent differences that are lower than average for interspecies comparisons and higher than average for intraspecies comparisons. They thus seem to represent recently diverged but separate taxa. The remaining species pairs are morphologically more similar and have individuals of each type with nearly identical genotypes, suggesting that they may be synonyms. A complete nomenclatural revision is in preparation (J.D. Williams, personal communication). Many of these pairs also failed to resolve as reciprocally monophyletic. The *Pleurobema cordatum* group (*P. cordatum*, *P. plenum*, *P. rubrum*, *P. sintoxia*) also is poorly resolved, but more intensive sampling throughout the Mississippi-Ohio-Tennessee river system is needed to understand this clade. Because it is both geographically and phylogenetically separate from the upper Coosa forms, we did not pursue them in detail. The molecular data and phylogenetic analyses thus suggest that current, morphological classification has slightly oversplit *Pleurobema*. But even if each of these species pairs were combined for conservation purposes, all the species would remain highly imperiled. Moreover, current nomenclature and literature fail to capture the diversity of supraspecific clades. Present data indicate higher levels of endemism than previously recognized, with species and higher clades generally each confined to a single river system. High endemism within drainages is also

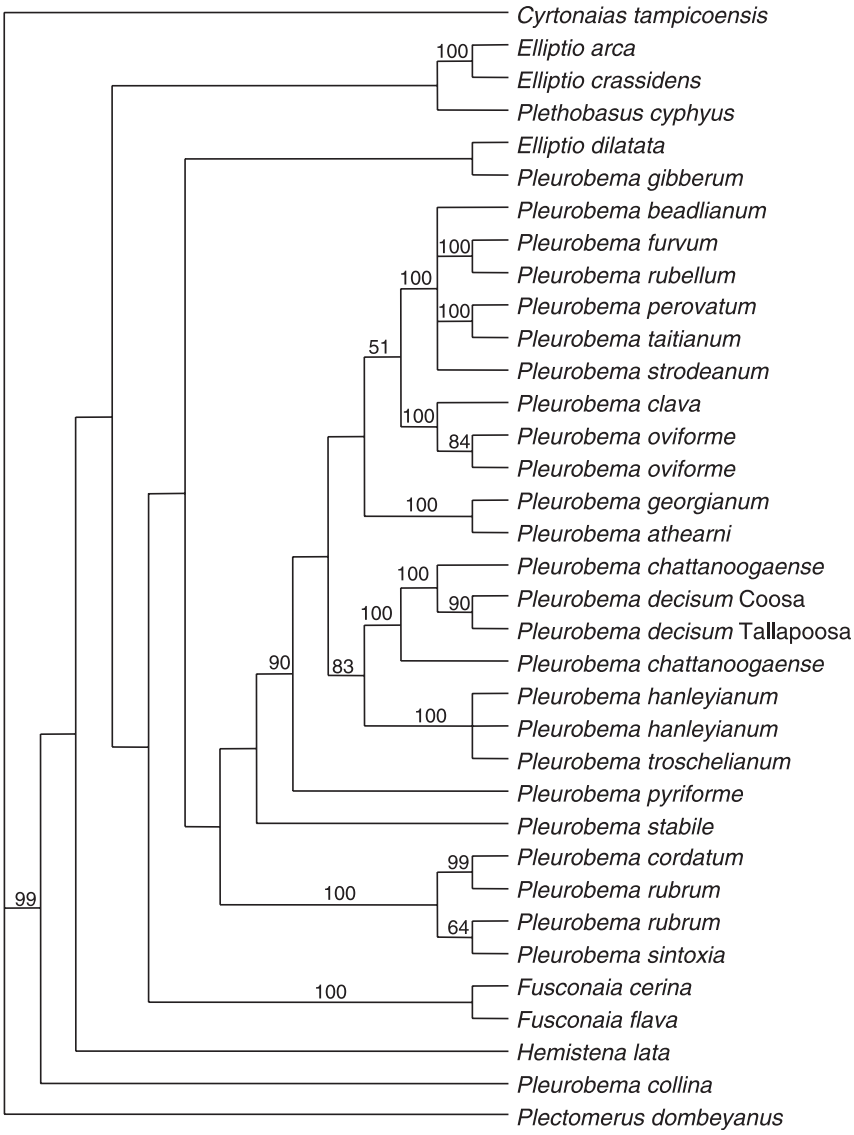

Fig. 6 Strict consensus of four trees, length 1473, *nadh1* and *cox1* data. Numbers indicate bootstrap percentage if over 50%.

increasingly recognized in the fish of the region (Boschung & Mayden 2004).

The present results reflect both the potential and the pitfalls of molecular barcoding approaches (Smith 2005). Molecular data provided key evidence that one unknown specimen was a different species from all other sampled species, leading to its recognition as *P. stabile*, and other problematic specimens were readily assigned to species based on DNA sequence. However, identification of the species required careful morphological studies to supply reliable reference DNA sequences. The lack of molecular data for many of the rarest species (especially other, probably extinct forms) make morphological examination of voucher material essential to identification of unusual specimens (De Ley *et al*. 2005).

Nadh1 yielded results almost identical to those for *cox1* for barcoding purposes and only a few differences in the phylogeny when analysed separately. Given the apparent fixed differences between male and female mitotypes in unionids (Hoeh *et al*. 2002; Curole & Kocher 2005), all mitochondrial genes are expected to show similar evolutionary patterns. As *nadh1* has a slightly higher level of interspecies variability and slightly lower intraspecies variability than *cox1*, it might be a better barcode choice than *cox1* in unionids. However, Jones *et al*. (2006) showed that mitochondrial sequence data alone (as in Buhay *et al*. 2002, a previous study on the same taxa) does not capture some of the species diversity in unionids. Grobler *et al*. (2007) found apparent mitochondrial introgression or ancestral polymorphism, indicating further risks for reliance solely on a

mitochondrial barcode. ITS1 showed a lower percentage variation and poor resolution, with some discrepancies with the mitochondrial data. ITS1 often has multiple alleles within a single individual (Campbell *et al*. 2004), leading to the potential of reticulate evolution through recombination between multiple ancestral alleles, lineage sorting, or other confounding effects. The lack of variation in ITS1 sequence within the *P. decisum-P. chattanoogaense* clade suggests that the region has undergone concerted evolution occasionally within the Unionidae, but the lack of clear pattern in most other sampled species suggests that such events have been infrequent within Pleurobemini. Unfortunately, better nuclear genetic markers have not yet been identified for most mollusks. The degree of variation shown in each sequence region varied from taxon to taxon. This supports cautions about the reliability of genetic barcoding that uses a single DNA region and a universal percentage cut-off for species recognition (Hickerson *et al*. 2006; Meier *et al*. 2006; Nielsen & Matz 2006). Nevertheless, the present barcode data set provides a powerful identification tool for the often problematic species in *Pleurobema*.

The high level of endemism has important implications for conservation. Although preservation of the few remaining localities with good faunas is crucial, focus on only the most diverse faunas will fail to protect many species. Restoration and protection of habitat in each of the river systems is necessary to protect all the taxa. In particular, incorrectly treating *P. stabile* and *P. hanleyianum* as synonyms of species found in other drainages could allow the Coosa species to go extinct under the mistaken belief that they were protected elsewhere. Also, the evolutionary lineages identified in this study may influence conservation decisions. Given the unfortunate reality of limited conservation funding, some authors propose prioritizing phylogenetically distinctive taxa (Vane-Wright *et al*. 1991; Faith 1992). The genetic distinctiveness of *P. stabile* would make its conservation a high priority.

The rediscovery of these species provides a new opportunity for their conservation. Because they were presumed to be extinct, none of these species currently have any legal protection. Reviews of their status and proposals to add them to the Endangered species list are in preparation. The taxonomic confusion that existed before our analyses hindered assessment of conservation needs. The present data provide better justification of alpha taxonomy and molecular tools to help in identification. Also, preservation of genetic diversity within a species provides greater evolutionary resiliency and avoids inbreeding problems. The concentration of genetic diversity in *P. decisum* (including *P. chattanoogaense*) in the remnant upper Coosa population suggests that this region is exceptionally important to the total diversity of the species.

The discovery of living individuals of several *Pleurobema* species raises some hope of preserving them from extinction if prompt efforts are made to protect their environment. Habitat restoration in the upper Coosa system, such as establishment of riparian buffer zones or restoration of a more natural flow regime below dams, would provide natural or restocked populations with better opportunities to survive and recover. Without such changes, the future of these species will be tentative at best. Regulation of point-source pollution has already ameliorated water quality. The Conasauga River, a Coosa tributary, was known a few decades ago as the 'Rainbow River' because its colour constantly varied because of factories discharging waste dyes. Such dramatic insults are gone, but the subtler effects of nonpoint pollution, excessive siltation, and unchecked suburban sprawl could easily eliminate the few survivors. For *P. stabile*, searches of recent collections yielded only one other specimen, also badly eroded externally, collected as a freshly dead shell. No more specimens have been found since 2001, live or dead. The situation for *P. hanleyianum* (whether or not *Pleurobema troschelianum* is treated as a synonym) is not much better, last found freshly dead in 2003. Mussels may live for decades, so a slowly dwindling, nonreproducing population may exist long after it is no longer self-perpetuating (Strayer *et al*. 2004). Also, the limited legal protection of the river systems and high (and increasing) anthropogenic impact lead to continuing habitat degradation. Although the Conasauga River has the highest remaining concentration of severely imperiled species in the upper Coosa system (eight Federally listed species, one candidate for Federal listing, and several species either endemic or extirpated from all other localities), it has received little conservation attention. Much of the historic range in the Coosa River system is now unsuitable habitat due to impoundment, unnatural flow regimes caused by inadequately regulated hydroelectric dam releases, siltation from poor land use and other detrimental modification (Burkhead *et al*. 1997; Mirarchi *et al*. 2004; Gangloff & Feminella 2007; Poff *et al*. 2007). In drought years, water demand from growing urban centres, especially Atlanta, poses a new threat. Almost the entire upper Coosa system lies within 150 km of Atlanta, putting many species at high risk of disturbance throughout their range. Competition by the introduced Asian clam, *Corbicula leana* (Prime 1864), may also affect the Coosa bivalve fauna. Most current threats could be reduced by proactive planning and better watershed practices. However, freshwater mollusks seem highly vulnerable to the effects of global warming (Mouthon & Daufresne 2006), making international as well as regional action important.

Similar threats face freshwater systems worldwide. As a result, nonmarine mollusks rank globally among the most imperiled organisms (Lydeard *et al*. 2004). The rediscovery of multiple species on the brink of extinction highlights the urgent need for protection and study of freshwater faunas,

especially in areas of high endemism such as southeastern North America.

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