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Phylogeographic analysis of the threatened and endangered superconglutinate-producing mussels of the genus *Lampsilis* (Bivalvia: Unionidae)

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**Abstract**

Several species of freshwater unionid mussels in the genus *Lampsilis* exhibit a remarkable reproductive strategy. Female mussels of these species enclose their larvae in a minnow-like lure, called a ‘superconglutinate’, to attract piscivorous fishes. When a fish attempts to ingest the superconglutinate the lure ruptures and the larvae are released to parasitize the fish. Of the four species of mussel which exhibit this strategy and are endemic to the Gulf Coast drainages of the southeastern United States, three are protected under the Endangered Species Act, and one is recognized as imperilled. Phylogenetic analysis of nucleotide sequences of the mitochondrial 16S ribosomal RNA and the first subunit of the cytochrome oxidase c genes was conducted on 18 individual specimens representing these four species and six outgroup taxa. Phylogenetic analyses of these data support the monophyly of the superconglutinate-producing mussels, and indicates a strong geographical component to the data. The zoogeographic patterns of the four taxa included in the study are congruent with those seen in freshwater vertebrates, and are consistent with a vicariant pattern resulting from fluctuations in sea level during the Pleistocene. Despite the strong geographical structuring of the data, only one species, *Lampsilis subangulata*, was recovered as monophyletic. The authors attribute the lack of support for the monophyly of the remaining species to insufficient sequence variation and the recent origin of the ancestor of these taxa. Based on these data, any future captive breeding projects aimed at augmenting or re-establishing populations should do so only from the appropriate source populations so as to maintain the genetic integrity of these nascent species.

**Keywords**: fresh water, *Lampsilis*, mitochondrial DNA, phylogeny, phylogeography, Unionidae

Received 6 February 2001; revision received 1 June 2001; accepted 1 June 2001

**Introduction**

The southeastern United States has been recognized as one of the most biotically diverse regions in the world (Benz & Collins 1997), particularly for aquatic organisms (Lydeard & Mayden 1995). Freshwater mussels (Bivalvia: Unionoida) are no exception, the regional fauna includes approximately 100 species, many of which are endemic (Williams & Butler 1994). Sadly, this region of the United States also holds one of the most endangered faunas in the world (Benz & Collins 1997; Master et al. 1998). Whereas the historical and environmental factors that contributed to the origin of this diversity are not yet entirely understood, evidence for the cause of the recent decline in diversity points towards anthropogenic factors (Neves et al. 1997). Unionid mussels are among the most endangered organisms in North America (Williams et al. 1993; Stein & Flack 1997; Master et al. 1998); however, very few published studies have examined the genetic diversity of freshwater mussels at or below the species level (Li et al. 1996; Mulvey et al. 1997; Roe & Lydeard 1998; King et al. 1999; Lydeard et al. 2000; Turner et al. 2000). Understanding how genetic diversity of threatened or endangered organisms is distributed geographically can provide insight into factors influencing the formation and maintenance of species and is critical to their conservation.
In most unionid species, the mature larvae, called glochidia, are obligate parasites on vertebrate hosts, typically fishes (McMahon 1993). Many species of Unionidae exhibit behaviours and structures that are presumably involved in maximizing the likelihood that their larvae will encounter a suitable host (Kat 1984; Barnhart & Roberts 1997). For example, some freshwater mussels discharge glochidia in conglutinates, mucous-like packages that resemble small worms or fish larvae (e.g. Kat 1984; Hartfield & Hartfield 1996), and entice a potential host with the prospect of food.

The freshwater mussel genus _Lampsilis_ includes 32 North American taxa (Turgeon et al. 1998), many of which use some type of lure to attract a host. In the majority of species of _Lampsilis_, a lure is formed by pigmented portions of the inner lobe of the mantle margin, which when extended mimic a small fish (Barnhart & Roberts 1997). The illusion of a swimming fish is heightened by the wave-like movements of the mantle which originate at the anterior end and move posteriorly toward the incurrent aperture. A more complete description of these mantle margins can be found in Kramer (1970).

Another distinctly different type of piscine lure has recently been described for four species of _Lampsilis_. In these taxa the glochidia are packaged in conglutinates, but because these lures are composed of many such packages they are referred to as superconglutinates (Haag et al. 1995). The superconglutinate mass is extruded from the excurrent aperture of the mussel in a transparent mucous tube that reaches up to 250 cm in length (Haag et al. 1995; Hartfield & Butler 1997). Water currents act on the mucous strand and cause the lure to mimic the swimming movements of a small fish (Fig. 1). The species known to produce superconglutinate lures are: _Lampsilis altilis_ (Conrad), _L. perovalis_ (Conrad), _L. australis_ (Simpson) (H. Blalock-Herod, personal comm.) and _L. subangulata_ (L. Lea) (Haag et al. 1995, 1999; O’Brien et al. 1997).

These four species are distributed in Alabama, Florida and Georgia (Fig. 2). The historic ranges of _L. altilis_ and _L. perovalis_ consisted of the Alabama and Black Warrior/Tombigbee river systems that together constitute the Mobile River Basin. Both species were described from specimens collected at the same location on the Alabama River (Conrad 1834). Presently, the range of _L. altilis_ is reported to be limited to portions of the Alabama River System (Haag et al. 1999), while _L. perovalis_ is considered to be restricted to the headwaters and tributaries in the Black Warrior/Tombigbee river systems (U.S. Fish & Wildlife Service 1994). Both of these taxa are listed as threatened species by the U.S. Fish & Wildlife Service (U.S. Fish & Wildlife Service 1994). _L. australis_ is known historically from the Choctawhatchee River System and the Yellow and Escambia river systems (Clench & Turner 1956), and is recognized as an imperilled species (Lydeard et al. 1999). _L. subangulata_ has been described from the Choctawhatchee River and the Apalachicola River System, which consists of the Flint and Chattahoochie rivers. The range of both of these species has been greatly reduced (Roe, KJ, personal observation; O’Brien & Brim-Box 1999), and _L. subangulata_ is listed as a federally endangered species (U.S. Fish & Wildlife Service 1998).

Knowledge of phylogenetic relationships and how genetic diversity is partitioned geographically is crucial to establishing conservation priorities for unionids (Lydeard & Roe 1998; King et al. 1999). In this study, we use the DNA sequences of two mitochondrial genes to test the monophyly and assess the phylogeographic structure of the superconglutinate-producing mussels. We also interpret the phylogeographic patterns recovered in this study in light of the geological history of the Gulf region and compare
these results to zoogeographic patterns observed in other freshwater taxa in the southeastern United States.

Materials and methods

Nucleic acid isolation, polymerase chain reaction and sequencing

Specimens of the four taxa in question were obtained from throughout the respective species ranges (Table 1). Protected species were collected by permission of the U.S. Fish & Wildlife Service (permit no. SA96–31). Because of the protected status and rarity of these species, the sample sizes used in this study are necessarily small. The inclusion of additional freshwater mussel taxa is based on previous phylogenetic studies of higher order relationships, which included representatives of all lampsiline genera (K. J. Roe, unpublished PhD dissertation). Voucher specimens were deposited at The University of Alabama Unionid Collection (UAUC). Taxonomic nomenclature used follows that employed in Turgeon et al. (1998).

Because of the potential for amplification of non-orthologous DNA sequences, as unionid mussels exhibit bi-parental inheritance of mitochondria (Hoeh et al. 1996), only somatic tissues were used. Total DNA was isolated from either fresh dead, frozen, or ethanol-preserved specimens using a standard phenol–chloroform method (Palumbi et al. 1991). Double-stranded and single-stranded DNA was generated via the polymerase chain reaction (PCR) using the primers LCO1490 and HCO2198 (Folmer et al. 1994) for the first subunit of the cytochrome oxidase c gene (COI), and 16SrL-myt and 16SrH-myt (Lydeard et al. 1996) for the 16S ribosomal RNA (rRNA) gene. PCR and manual DNA sequencing protocols follow Roe & Lydeard (1998) for the COI gene portion and Lydeard et al. (1996) for the 16S rRNA portion. Sequencing of both heavy and light strands for some taxa was performed using Big Dye (Perkin Elmer) terminator cycle sequencing and the products were visualized using an ABI 377 automated sequencer.

Analysis of sequence data

COI sequences were aligned by eye using the software package xeyes (Cabot & Beckenbach 1989) and 16S rDNA sequences were aligned using clustalw (Thompson et al. 1994). Aligned matrices are available from the first author. No insertions–deletions (indels) were observed in the COI data set. Phylogenetically informative indels in the 16S data set were coded as binary characters and the resulting matrix was appended to the aligned data set. Prior to
phylogenetic analysis, the DNA sequences were examined for the presence of significant phylogenetic signal using the $g$-shape parameter ($g$-statistic (Hillis & Huelsenbeck 1992). Sequences were tested for the parametric pairwise parsimony (parametric pairwise parsimony) under the HKY 85 model (Hasegawa et al. 1985). Based on the results of the parametric pairwise parsimony analysis only. Estimates of sequence divergence were made using the HKY 85 model (Hasegawa et al. 1985). Alternative topologies (i.e. constraining the monophyly of a particular group) were compared statistically to the optimal trees for each analysis using the nonparametric pairwise parsimony test proposed by Templeton (1983) as implemented in PAUP* 4.0b4a. In the instance where multiple equally parsimonious trees resulted from constraining the analysis, a single representative tree was used for the Templeton test. The phylogeography of the group was investigated using area cladograms derived using Brooks Parsimony Analysis (BPA) (Wiley 1988). The resulting pattern was examined for congruence with existing area relationship hypotheses for the region.

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Table 1 Localities and GenBank accession numbers of specimens used in this study

| Lamplus altilis #1 – Chewacla Creek, Tallapoosa River Drainage, Macon Co., AL, UAUC #248; AF385092, AF385116 |
| L. altilis #3 – Little Cahaba River, Cahaba River Drainage, Jefferson Co., AL, UAUC #149; AF385108, AF385132 |
| L. altilis #4 – Fish Creek, Coosa River Drainage, Polk Co., GA, UAUC #650; AF385107, AF385131 |
| L. altilis #5 – Conasauga River, Coosa River Drainage, Murray/Whitfield Co., GA, UAUC #651, AF385106, AF385130 |
| L. australis #1 – West Fork of Chocotawhatchee River, Barbour Co., AL, UAUC #128, AF385101, AF385125 |
| L. australis #2 – West Fork of Chocotawhatchee River, Barbour Co., AL, UAUC #130, AF385098, AF385122 |
| L. australis #3 – Flat Creek, Pea River Drainage, Geneve Co., AL, UAUC #547, AF385097, AF385121 |
| L. australis #4 – Shoal River, Yellow River Drainage, Okaloosa Co., FL, UAUC #643, AF385100, AF385124 |
| L. australis #5 – Conecuh River, Escambia River Drainage, Pike Co., AL, UAUC #510, AF385099, AF385123 |
| L. perovisi #1 – Lubbb Creek, Tombigbee River Drainage, Pickens Co., AL, UAUC #86; AF385093, AF385117 |
| L. perovisi #2 – North River, Black Warrior River Drainage, Tuscaloosa Co., AL, UAUC #107; AF385094, AF385118 |
| L. perovisi #3 – Sipsey River, Tombigbee River Drainage, Greene/Pickens Co., AL, UAUC #646; AF385096, AF385120 |
| L. perovisi #4 – Brown Creek, Black Warrior River Drainage, Winston Co., AL, UAUC #648; AF385095, AF385119 |
| L. perovisi #5 – Flannigan Creek, Black Warrior River Drainage, Lawrence Co., AL, UAUC #649; AF385091, AF385115 |
| L. subangulata #1 – Kinchafloon Creek, Flint River Drainage, Webster Co., GA, UAUC #133; AF385102, AF385126 |
| L. subangulata #2 – Uchee Creek, Chattahoochee River Drainage, Russell Co., AL, UAUC #116; AF385104, AF385128 |
| L. subangulata #3 – Whitewater Creek, Flint River Drainage, Fayette Co., GA, UAUC #645; AF385103, AF385127 |
| L. erinata – Cahaba River, Alabama River Drainage, Bibb Co., AL, UAUC #17, AF385112, AF385136 |
| L. enta – Elk River, Tennessee River Drainage, Limestone Co., AL, UAUC #108; AF385111, AF385135 |
| L. trifida – Yellowleaf Creek, Coosa River Drainage, Shelby Co., AL, UAUC #6; AF385113, AF385137 |
| L. recta – Ohio River, near Louisville, KY, UAUC #89; AF385110, AF385134 |
| L. ornata – Original Suwanee River campground canals, Suwanee River Drainage, Dixie Co., FL, UAUC #652, AF385109, AF385133 |
| Oligobranchus reflexa – Cahaba River, Alabama River Drainage, Bibb Co., AL, UAUC #19, AF385114, AF385138 |

UAUC = University of Alabama Unions Collection.

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Results

Nucleotide variation and saturation

DNA sequences for 24 taxa resulted in a data matrix of 590 characters for the COI gene, and 432 (410 nucleotides + 22 indels) characters for the 16S rRNA gene, for a total of 1022 characters. The COI data set contained 169 variable sites, 92 of these were phylogenetically informative; whereas the 16S data set contained 112 variable sites, 68 of which were phylogenetically informative. Preliminary analysis of DNA sequences revealed no evidence of saturation due to multiple substitutions for either gene portion. The $g^2$ statistic indicates the presence of a significant phylogenetic signal ($P = 0.01$) for the combined data set (COI + 16S = –0.581541). Pair-wise comparisons of sequence divergence using the HKY 85 model (Hasegawa et al. 1985) are presented in Table 2. Intraspecific distances ranged from 0.00–1.62% ($L. altilis$) to 0.30–2.82% ($L. australis$). Within the superconglutinate clade, interspecific comparisons ranged from 0.31–2.42% ($L. altilis$ – $L. perovalis$) to 3.74 – 5.45% ($L. perovalis$ – $L. australis$).

Phylogenetic analysis

Analysis of combined 16S and COI data under MP resulted in 24 equally parsimonious trees of 501 steps (CI = 0.649,
All of the superconglutinate-producing species (L. altilis, L. australis, L. perovalis and L. subangulata) were recovered as a monophyletic group in all reconstructions. The analyses place Villosa villosa as the sister taxon to the superconglutinate producer clade. All topologies also show two sister clades (L. perovalis + L. altilis) and (L. australis + L. subangulata). Only L. subangulata is consistently recovered as monophyletic, although its placement as sister group to L. australis from the Pea-Choctawhatchee system renders L. australis paraphyletic. Trees resulting from constraining L. australis to be monophyletic are only two steps longer, and are not significantly worse than the optimal trees (Table 3).

Discussion

Monophyly of superconglutinate producers

The results of all phylogenetic analysis support the monophyly of the four superconglutinate producers and indicate a single origin of the superconglutinate lure in the common ancestor of these species. Support/decay values for the most parsimonious solutions indicate that 11 additional steps are required to collapse the node supporting
Phylogeography of superconglutinate producers

The Mobile Basin is one of the most biotically diverse freshwater systems in North America, containing many endemic species of aquatic organisms (Lydeard & Mayden 1995; Benz & Collins 1997). Gulf coastal drainages to the east of the Mobile Bay include the faunistically rich Apalachicola River System and the Escambia, Yellow and Choctawhatchee rivers. Several researchers have suggested that the distributions of species in this region are related to geological events that have altered drainage patterns (Wiley & Mayden 1985; Bermingham & Avise 1986; Swift et al. 1986). Examination of the area relationships indicated by taxa in this study reveals the following pattern (Fig. 3b): the Choctawhatchee River System is sister to the Apalachicola River System, which together are sister to the Mobile and Escambia rivers. All of these rivers together are sister to the Mobile System. The pattern of a split between the Mobile Basin and the rivers to the east is generally congruent with hypotheses derived from biogeographic analysis of fishes (Wiley & Mayden 1985; Swift et al. 1986; Kristmundsdóttir & Gold 1996; Wiley & Hagen 1997), and turtles (Lamb et al. 1994; Roman et al. 1999). All of these studies have sought to explain present day distributions of vertebrate taxa in Gulf Coastal Plain rivers using vicariant scenarios related to fluctuations in sea level during the latter part of the Cenozoic Era. The results of this study, although not strictly concordant with previously published studies of vertebrates, provides evidence of a similar phylogeographic pattern in freshwater bivalves.

Although it is impossible to determine accurately the time of origin for freshwater mussels in North America, fossil evidence indicates that unionid mussels may have been present in North America as early as the Triassic Period, at least 181 million years ago (Haas 1969). If the common ancestor of the superconglutinate-producing species was already present in the Gulf region before the Oligocene (37–24 Ma), the receding sea level during the Oligocene or the Late Miocene (10–5 Ma) or Pleistocene (2.8 to < 0.3 Ma) would have allowed connections and subsequent dispersal between the Mobile and Apalachicola systems and the intervening Coastal Plain rivers (Donn et al. 1962). Using the mtDNA divergence times derived from several species of fishes, Bermingham & Avise (1986) proposed that the split between the Mobile River and the Apalachicola and the Escambia rivers occurred approximately 750 000 years ago. Such a relatively recent divergence would support a Pleistocene origin of the ancestors of the (L. australis + L. perovalis) and (L. australis + L. subangulata) clades. A Pleistocene or Pleistocene origin has been suggested for other North American freshwater mussel genera (e.g. Haas 1969; Davis et al. 1981), and the fluctuations in sea level during these periods could have provided avenues for dispersal and the isolating mechanism that created the phylogenetic pattern recovered in this study. A Pleistocene origin for these species is also supported by the relatively low genetic divergences observed within and between species of superconglutinate producers (Table 2).
The results of this study confirm the monophyly of the superconglutinate-producing species of the genus *Lampsilis*. Although the data presented here do not unambiguously support the monophyly of three of the four species in this clade, the phylogeographic pattern, strong geographical partitioning and low genetic divergences tend to support a relatively recent origin for these species. Further support for the recognition of these taxa as natural groups will require unionid-specific markers for more variable regions of the mitochondrial and nuclear genomes, which are currently under development.

**Conservation implications**

The strong geographical structure observed in the data, combined with the apparent recent origin of these species, offer some guidance for the conservation of these taxa. All four of the superconglutinate-producing species are currently given some protection by either state or federal laws, and no captive rearing and propagation of any of these species is currently planned. The genetic data indicate that if such projects were to be instituted, care should be taken to maintain the genetic integrity of these nascent species by only augmenting existing populations, or re-establishing extirpated populations from the appropriate geographical source. In addition, monitoring of the numbers of each of these species should be conducted through regular surveys of the various watersheds. Recent surveys of freshwater mussels in the Mobile, Apalachicola, Conecuh and Pea/Choctawhatchee rivers revealed the complete absence of species (McGregor 2000a), restricted distribution of few individuals (McGregor 2000b), or found no evidence of any future efforts to restock species through captive rearing and propagation. Ultimately, the persistence of these species and the success of any future efforts to restock species through captive breeding will be dependent on the availability of suitable habitat throughout their respective ranges.

**Acknowledgements**

Thanks are extended to H. Blalock-Herod, J. Brim-Box, J. Garner, B. Butcher, M. Hughes, A. Lydeard, H. McCullagh, M. Pierson, A. M. Simons and J. D. Williams for providing specimens. J. Brim-Box and C. O’Brien kindly provided the superconglutinate photo. R. Butler, D. Grat, J. Serb and several anonymous reviewers provided information and helpful comments. This study was supported by the Department of Biological Sciences and the Graduate School of The University of Alabama, by funds from the U.S. Department of the Interior (#1448-40181–07-G-035), and the National Science Foundation (DEB-9707623) to Charles Lydeard. Protected mussels were collected under federal permit SA96-31.

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PHYLOGEOGRAPHY OF SUPERCONGLUTINATE PRODUCERS


Kevin J. Roe is a postdoctoral researcher at the University of Alabama. His research interests include the systematics and biogeography of freshwater molluscs and other aquatic organisms. Paul D. Hartfield has conducted numerous status surveys of freshwater bivalves in the Mobile basin and has written recovery plans for two of the species included in this study. Dr Charles Lydeard is working on molecular phylogenetics, conservation genetics and molecular evolution of nonmarine molluscs. All laboratory and data analyses were performed by the first author in the laboratory of Dr Lydeard.