

PATTERNS OF GENETIC DIFFERENTIATION AND CONSERVATION OF THE SLABSIDE PEARLYMUSSEL, *LEXINGTONIA DOLABELLOIDES* (LEA, 1840) IN THE TENNESSEE RIVER DRAINAGE

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(Received 26 October 2004; accepted 29 April 2005)

ABSTRACT

The restoration and recovery of imperiled mussel species will require the re-establishment of populations into historically occupied habitats. The possible existence of genetic differentiation among populations should be considered before inter-basin transfers are made. Eighty individuals of the federal candidate species *Lexingtonia dolabelloides* were sampled from populations in the North Fork Holston, Middle Fork Holston, Clinch, Paint Rock and Duck rivers of the Tennessee River basin in the southeastern United States. We sequenced 603 base-pairs of a mitochondrial DNA gene (*ND-1*) and 512 base-pairs of a nuclear DNA gene (*ITS-1*). Analyses of molecular variation (AMOVA) values for both genes indicated that the majority of variation in *L. dolabelloides* resided within populations (82.9–88.3%), with 11.7–17.1% of variation among populations. Haplotype frequencies differed significantly among populations for both genes sequenced. Clustering of haplotypes in minimum-spanning networks did not conform stringently to population boundaries, reflecting high within-population and low between-population variability. Maximum parsimony analysis did not identify any population as a monophyletic lineage. A Mantel test showed no significant correlation between geographical stream distance and genetic distance, thus not supporting a pattern of isolation-by-distance. Overall, results provided support to manage fragmented populations of *L. dolabelloides* in the Tennessee River drainage as two management units (MUs), but did not provide evidence for the existence of ESUs following published molecular criteria.

INTRODUCTION

The slabside pearlymussel, *Lexingtonia dolabelloides* (Lea, 1840), inhabits river shoals of coarse gravel and sand in medium to large streams and rivers. This species is a short-term summer brooder (tachytictic) that typically spawns and releases larvae (glochidia) from May to August. Various species of minnows (family Cyprinidae) probably serve as host fish (Parmalee & Bogan, 1998). The maximum age for the species is known to exceed 40 years (J. Jones, unpublished data).

It is estimated that the species has been eliminated from three-fifths of the total number of streams from which it was historically known in the main channels of the Cumberland and Tennessee rivers (USFWS, 1999). Currently, this mussel species is limited to fragmented and isolated populations in eight streams in the Tennessee River system: the Clinch, Powell, Elk, Duck, Hiwassee, North Fork and Middle Fork Holston, and Paint Rock rivers (Parmalee & Bogan, 1998). Current and historical distribution is summarized in Figure 1, from data by Parmalee, Klippel & Bogan (1980), Neves & Zale (1982), Ahlstedt (1983), Parmalee (1988), Starnes & Bogan (1988), Gordon & Layzer (1989), Layzer, Gordon & Anderson (1993), Parmalee & Hughes (1994), Parmalee & Bogan (1998), USFWS (1999), McGregor & Garner (2004) and Parmalee & Polhemus (2004). Primary causes of extirpation have been impoundments, water pollution, sedimentation and channel modifications (Williams *et al.*, 1992; Neves, 1993; Neves *et al.*,

1997). Populations continue to decline in many areas throughout the current range. Because of population losses, range reductions and declines in population abundance, *L. dolabelloides* was listed as a candidate species proposed for possible threatened or endangered status in 1999 (USFWS, 1999).

Widespread degradation or elimination of freshwater habitats has led to an emphasis on determining the genetic structure of freshwater snail and mussel species (Mulvey *et al.*, 1997; Machordom *et al.*, 2003; Hurt, 2004; Mock *et al.*, 2004). The concept and implementation of the Evolutionarily Significant Unit (ESU) framework has been the subject of much discussion and controversy (Nielsen, 1995). When planning re-introductions, a key first question is which populations may be mixed. Populations within many species may be sufficiently differentiated that they deserve management as separate units, because of historical isolation (Moritz, 2002) or because they are adapted to somewhat different environments (Frankham, Ballou & Briscoe, 2002). Moritz (2002) set specific molecular criteria for the recognition of evolutionarily significant units: ESUs should be reciprocally monophyletic for mtDNA and show significant divergence of allele frequencies at nuclear loci. Moritz (1994) also recognized populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of alleles, as management units (MUs). Waples (1991) proposed that, to qualify as an ESU, a population must be reproductively isolated from other conspecific units and represent an important component of the evolutionary legacy of the species. As an alternative to ESUs, Crandall *et al.* (2000) suggested that populations be classified

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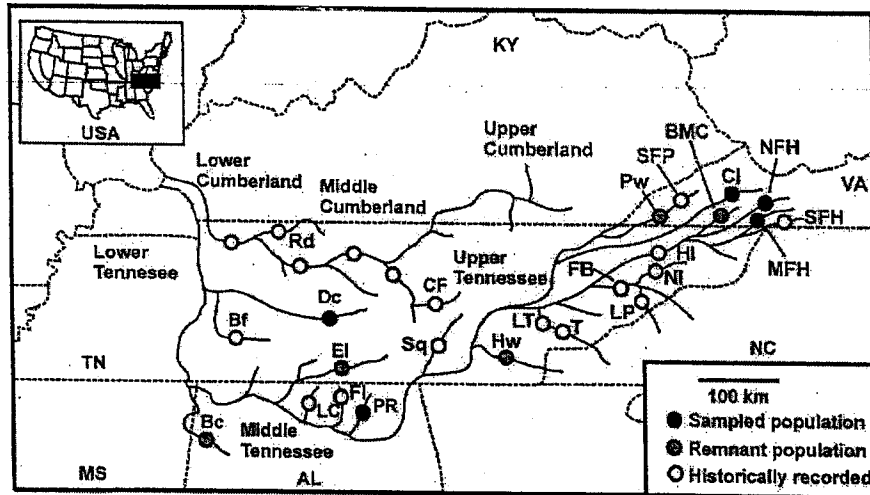


Figure 1. The distribution of collection localities used during the current study, smaller remnant populations and historical distribution areas for *Lexingtonia dolabelloides* in the Tennessee River drainage. Abbreviations used: BC, Bear Creek; Bf, Buffalo; BMC, Big Moccasin Creek; CF, Caney Fork; Cl, Clinch; Dc, Duck; El, Elk; FB, French Broad; Fl, Flint; HI, Holston; Hw, Hiwassee; LC, Limestone Creek; LP, Little Pigeon; NI, Nolichucky; LT, Little Tennessee; MFH, Middle Fork Holston; NfH, North Fork Holston; PR, Paint Rock; Rd, Red; SFP, South Fork Powell; SFH, South Fork Holston; Sq, Sequatchie; T, Tellico. States: AL, Alabama; KY, Kentucky; MS, Mississippi; NC, North Carolina; TN, Tennessee; VA, Virginia.

by recent or historical ecological or genetic exchangeability. These approaches attempt to determine whether there is adaptive differentiation among populations and whether there is historical or recent gene flow. Assessment of which populations or assemblages of populations comprise ESUs or MUs will facilitate the decision of which populations should be subject to conservation management, including the possibility of translocation or captive propagation and stocking of cultured individuals into the wild.

The objective of the current study was to investigate and quantify patterns of genetic differentiation in *L. dolabelloides*, so that any population subdivision can be considered when planning future restoration and recovery programmes.

MATERIAL AND METHODS

Sample localities and collection

Mussels were sampled from the following locations throughout the range of *L. dolabelloides* (Fig. 1): North Fork Holston River, Smyth Co., Virginia [a small but declining population (Jones & Neves, 2004)]; Middle Fork Holston River, Washington Co., Virginia [a large but declining population (Henley *et al.*, 2000)]; Clinch River, Russell Co., Virginia [a small but stable population (Ahlstedt, 1991a)]; Paint Rock River, Jackson Co., Alabama [a small but declining population (Ahlstedt, 1995)]; and Duck River, Maury Co., Tennessee [a large and stable population (Schilling & Williams, 2002; Ahlstedt *et al.*, 2004)].

Sample sizes were relatively low because of the imperiled status of some populations (see Tables 1 and 2, bearing in mind that both genes could not be sequenced in all specimens collected). No sampling was done from the Elk, Hiwassee and Powell rivers, since individuals are very rare in these remnant populations. A small piece of mantle tissue (20–50 mg) was collected non-lethally from each mussel sampled. Tissue was preserved in 95% ethanol prior to DNA isolation.

Genetic analysis

Total DNA was isolated using either the Roche Diagnostics® High Pure Template Preparation Kit or the Purgene® DNA

extraction kit. Success and quality of DNA extraction was verified using 0.8% agarose gels. Concentration of DNA extracts was quantified by fluorescence assay.

One mitochondrial DNA (mtDNA) region (*ND-1*) and one nuclear DNA (nDNA) region (*ITS-1*) were sequenced to determine variation among populations. We used primers and polymerase chain reaction (PCR) amplification conditions as reported in the following sources: (1) *ND-1*, first subunit of NADH dehydrogenase (Buhay *et al.*, 2002; Serb, Buhay & Lydeard, 2003), and (2) *ITS-1*, first internal transcribed spacer region between the 5.8S and 18S ribosomal DNA genes (King *et al.*, 1999). Primer sequences were *ND-1* forward: AAAAAGCTTCCGTAGGTGAACCTG; *ND-1* reverse: AGC TTGCTGCGTTCTTCATCG; *ITS-1* forward: TGGCAGAAA AGTGCATCAGATTAAGC; and *ITS-1* reverse: TCGGAAT TCTCCTTCTGCAAAGTC.

The PCR reaction mixture for *ND-1* consisted of 100 ng of genomic DNA, 1 × PCR buffer, 4.0 mM MgCl₂, 0.4 mM dNTPs, 1.0 mM each primer, and 1.5 U AmpliTaq Gold DNA polymerase, with ddH₂O added to a total volume of 50 µl. A total reaction volume of 25 µl gave similar results. Conditions for amplification were: an initial 95°C for 8 min; followed by 35 cycles of: 94°C for 40 s, 50°C for 60 s and 72°C for 90 s; with a final extension step at 72°C for 2 min; and a final hold at 4°C.

The PCR reaction mixture for *ITS-1* consisted of 100 ng of genomic DNA, 1 × PCR buffer, 2 mM MgCl₂, 0.25 mM dNTPs, 0.5 mM each primer and 1.0 U AmpliTaq DNA polymerase, with ddH₂O added to a total volume of 20 µl. Amplification conditions were: an initial 95°C for 7 min; followed by 35 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 90 s; with a final extension step at 72°C for 5 min; and a 4°C hold.

A similar DNA sequencing protocol was followed for both *ND-1* and *ITS-1*. PCR products first were purified using a Qiagen® DNA purification kit to remove any remaining primers. PCR products then were sequenced with a Big Dye Terminator Cycle Sequencing kit with AmpliTaq DNA Polymerase (Applied Biosystems®). The reaction mixture for sequencing reactions consisted of 3 µl Big Dye, 0.5 µl primer, PCR product (1 µl for *ITS-1* and 2 µl for *ND-1*); with

Table 2. Haplotypes (with indication of polymorphic sites), haplotype frequencies, shared haplotypes and indices of population diversity for *ITS-1* in five populations of *Lexingtonia dolabelloides* (dash denotes deletions).

	Haplotypes and polymorphic nucleotide sites:										Population:					
	2	2	6	6	2	3	3	3	3	4	4	North Fork (n = 10)	Middle Fork (n = 12)	Clinch (n = 8)	Paint Rock (n = 10)	Duck (n = 12)
	0	7	3	4	7	7	4	5	8	3	6					
1	A	A	-	A	-	T	T	-	-	C	-	0.917	0.417	0.333	0.900	0.357
2	C	A	.	.	0.083	-	0.111	0.100	0.071
3	.	.	.	C	-	0.333	0.111	-	-
4	A	.	.	-	0.083	-	-	-
5	.	.	.	C	.	.	.	C	A	.	.	-	0.167	0.222	-	-
6	G	.	-	-	0.111	-	-
7	G	-	-	0.111	-	-
8	C	-	-	-	-	0.214
9	.	.	A	.	.	G	-	-	-	-	0.071
10	C	G	.	-	-	-	-	0.071
11	-	-	-	-	0.143
12	.	.	.	C	-	-	-	-	0.071
Number of haplotypes:												2	4	6	2	7
Number of polymorphic sites:												2	3	5	2	8
Nucleotide diversity (%):												0.079 ± 0.089	0.247 ± 0.188	0.337 ± 0.249	0.079 ± 0.089	0.318 ± 0.227

ddH₂O added to a total volume of 15 ml. Cycling conditions were 35 cycles, each consisting of 94°C for 30 s, 50°C for 15 s and 60°C for 4 min; with a final holding temperature of 4°C. Products of sequencing reactions were resolved on ABI377 or ABI3100 automated sequencers. Sequencer-generated results were stored as GENESCAN files, and SEQUENCHER software (version 4.11) was used to align and edit sequences.

Statistical analysis

All analyses were done separately for results of the mtDNA marker (*ND-1*) and nDNA marker (*ITS-1*). DNA sequences first were summarized by identifying haplotypes and assigning each individual to a specific haplotype. We also calculated basic indices of population diversity, i.e. number of haplotypes, number of single nucleotide polymorphisms and nucleotide diversity (π), using ARLEQUIN software (Schneider, Roessli & Excoffier, 2000). The significance of differences between haplotype frequencies was assessed using a chi-squared test. Spatial distribution of genetic variation was assessed using an analysis of molecular variance (AMOVA) as implemented in ARLEQUIN, with sequence variation partitioned into within- and among-population components. One thousand permutations were used to provide significance tests for each of the variance components. For more specific measures of pairwise population differentiation, we calculated conventional F_{ST} indices from haplotypes (with related P values) and number of nucleotide substitutions. The Kimura (1980) two-parameter (K2P) model was considered appropriate for the *ND-1* data since transitional nucleotide substitutions outnumbered transversional substitutions. A similar pattern of substitution was not found for the *ITS-1* data; therefore, we used the model of Jukes & Cantor (1969) (J-C), which assumes equal frequencies of substitution. A Mantel test (Smouse & Long, 1992) was used to test for isolation by distance. We measured the correlation between K2P or J-C values and geographic stream distance, using PASSAGE, version 1.0.3.8 (Rosenberg, 2003).

Minimum-spanning networks were constructed based on the minimum number of nucleotide mutations between different haplotypes. The networks were constructed using NETWORK, version 4.1.0.0. software (www.fluxus-engineering.com), employing the median joining approach (Bandelt, Forster & Röhl, 1999). Phylogenetic analysis of the *ND-1* and *ITS-1* gene sequences was conducted using PAUP* version 4.1 (Swofford, 1995). All searches were based on maximum parsimony (MP) and employed full heuristic searches. We used 1,000 bootstrap replicates, and the proportion of bootstrap trees that resolved a branch was taken as the measure of support for that branch. Only values >50% were presented in the final phylograms. Sequences of *ND-1* and *ITS-1* genes from *Epioblasma capsaeformis* were used as the outgroup taxon to root trees. Choice of this species was based on the availability of sequences for these genes, generated in our laboratory and therefore based on identical methodology and with similar length compared to *L. dolabelloides*, which resulted in unambiguous alignment of sequences.

RESULTS

Results from mtDNA (*ND-1*)

We were able to consistently amplify 603 base-pairs (bp) for this mitochondrial gene. Thirty-one single nucleotide polymorphisms were found and 24 distinct haplotypes were identified (GenBank accession numbers AY773302-AY773326). Haplotypes resolved and the frequency of each haplotype is presented in Table 1. Examination of shared haplotypes in Table 1 does not show fixation of haplotypes among populations from the North Fork Holston, Middle Fork Holston, Clinch and Paint Rock rivers. However, the Duck River population shares only one haplotype with the other populations; haplotype '8', which is the most common haplotype in the Duck River population (29.4%), but was found in only one other mussel in other rivers, an individual from the Middle Fork Holston River.

Inspection of haplotypes in Table 1 suggests that haplotype frequency differences exist among most populations, with large

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numbers of unique haplotypes in each population. Results of a chi-squared test indicated that differences between haplotype frequencies were significant ($P = 0.002$). In many instances, a specific haplotype is unique not only among populations but also within a population (i.e. with a frequency of one individual). Results from AMOVA indicated that 82.9% of variation in *L. dolabelloides* resided within populations, with 17.1% among populations.

Since haplotypes at low frequency could, by chance, be sampled in one population but not another, we conducted a more conservative analysis, restricted to haplotypes observed in more than one individual. Using this approach, there was considerable overlap between the populations from the North Fork Holston, Middle Fork Holston, Clinch and Paint Rock rivers. Haplotype '1' occurred in 50% of the North Fork population, 43.8% of the Middle Fork mussels, 33.3% of the Clinch mussels and all Paint Rock individuals, suggesting relatively uninterrupted gene-flow among these four populations. However, this common haplotype is absent from the Duck River population, and the only overlap between the Duck River mussels and the remaining populations was for haplotype '8', as described above.

Using all haplotypes again, pairwise F_{ST} values indicative of significant ($P = 0.001-0.015$) differentiation were estimated between the Duck River population and all other populations (Table 3), as well as between the Paint Rock River population and all other populations ($P = 0.003-0.015$). No F_{ST} values among the upper Tennessee populations (North Fork Holston, Middle Fork Holston and Clinch) suggested significant differentiation ($P = 0.195-0.797$).

The number of nucleotide substitutions (K2P) between individuals within populations ranged from 0 to 5.62%, with an average of 2.52% (Table 3). Substitutions between populations (corrected for back mutations) ranged from 0.06% to 1.46%, and averaged 0.58%. Results from the Mantel test comparing K2P values with stream distance suggested that the correlation ($r = 0.315$) was not significant ($P = 0.199$). Patterns of nucleotide substitution and stream distance, therefore, did not reflect isolation-by-distance.

The minimum-spanning network constructed from *ND-1* haplotypes (Fig. 2A) did not group *L. dolabelloides* individuals along unambiguous population boundaries. This finding parallels the low level of among-population divergence seen from AMOVA results. There was, however, a grouping of Duck River individuals, with 16 of 17 animals sampled within 0-2 mutational steps from a single haplotype (haplotype '8'), and only one individual separated by three mutational steps from the rest. This grouping supported the hypothesis of some degree of genetic distinctiveness for the Duck River population. The Middle Fork Holston River population showed some degree of distinctiveness, with a maximum of three mutational steps between any haplotype and haplotype '8'. Populations from the North Fork Holston and Clinch rivers showed a high level of nucleotide variation, with up to 10 (North Fork Holston) and 13 (Clinch) mutational steps between haplotypes. In contrast, the *ND-1* haplotypes from the Paint Rock River population of *L. dolabelloides* were identical and fixed for haplotype 1.

Maximum parsimony analysis of the *ND-1* gene sequences yielded a single most parsimonious tree of 154 steps with a consistency index (CI) of 0.82. There were only three branches in the phylogram recognized with >50% bootstrap support (Fig. 3), and there were no groupings of populations in clades to support the inference that any population of *L. dolabelloides* was monophyletic relative to the other conspecific populations.

Nucleotide diversity within populations (Table 1) indicated loss of diversity in the Paint Rock River population, with no observed diversity. Values for other populations ranged from 0.29-0.52% in the North Fork Holston, Middle Fork Holston and Duck populations, with the most diversity (0.92%) in the Clinch River population.

Results from nuclear DNA (*ITS-1*)

A total of 512 bp were amplified for this nuclear gene, and 11 single nucleotide polymorphisms were observed. Twelve haplotypes were identified (GenBank accession numbers AY772175-AY772186). Haplotypes resolved, frequencies of each haplotype and a comparison of haplotypes shared among populations are

Table 3. Pairwise F_{ST} and corrected K2P/J-C values among five populations of *Lexingtonia dolabelloides*, from *ND-1* (below diagonal) and *ITS-1* (above diagonal). The nucleotide substitution values below locality names indicate average substitution (uncorrected) among individuals within populations.

	North Fork J-C = 0	Middle Fork J-C = 0.551	Clinch J-C = 0.866	Paint Rock J-C = 0	Duck J-C = 0.581
North Fork K2P = 3.143	-	$F_{ST} = 0.230$ ($P = 0.011$) J-C = 0.229	$F_{ST} = 0.211$ ($P = 0.041$) J-C = 0.072	$F_{ST} = -0.111$ ($P = 0.999$) J-C = 0.0	$F_{ST} = 0.169$ ($P = 0.020$) J-C = 0.046
Middle Fork K2P = 1.768	$F_{ST} = 0.026$ ($P = 0.797$) K2P = 0.064	-	$F_{ST} = -0.021$ ($P = 0.540$) J-C = 0.049	$F_{ST} = 0.230$ ($P = 0.010$) J-C = 0.229	$F_{ST} = 0.056$ ($P = 0.073$) J-C = 0.275
Clinch K2P = 5.621	$F_{ST} = 0.011$ ($P = 0.231$) K2P = 0.519	$F_{ST} = 0.072$ ($P = 0.195$) K2P = 0.974	-	$F_{ST} = 0.211$ ($P = 0.046$) J-C = 0.072	$F_{ST} = 0.008$ ($P = 0.295$) J-C = 0.118
Paint Rock K2P = 0	$F_{ST} = 0.198$ ($P = 0.015$) K2P = 0.192	$F_{ST} = 0.277$ ($P = 0.011$) K2P = 0.183	$F_{ST} = 0.331$ ($P = 0.003$) K2P = 1.459	-	$F_{ST} = 0.169$ ($P = 0.017$) J-C = 0.046
Duck K2P = 2.083	$F_{ST} = 0.191$ ($P = 0.001$) K2P = 0.364	$F_{ST} = 0.176$ ($P = 0.001$) K2P = 0.354	$F_{ST} = 0.106$ ($P = 0.002$) K2P = 1.236	$F_{ST} = 0.493$ ($P = 0.001$) K2P = 0.493	-

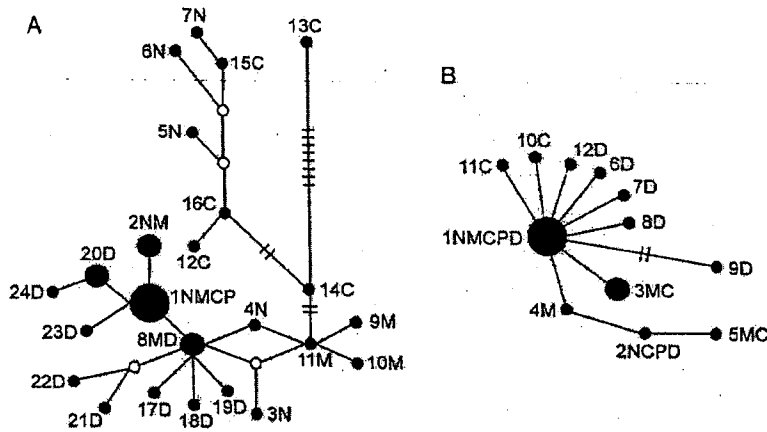


Figure 2. Minimum spanning networks between (A) 24 *ND-1* haplotypes and (B) 12 *ITS-1* haplotypes, observed in *Lexingtonia dolabelloides*. Each node represents a haplotype and the size of the node indicates the number of individuals sharing that haplotype. Notation of nodes: number = haplotype number (following Tables 1 and 2); letter = population(s) possessing the haplotype. Abbreviations: C, Clinch, D, Duck, M, Middle Fork Holston, N, North Fork Holston; P, Paint Rock.

presented in Table 2. Examination of shared haplotypes showed no fixation for different sets of haplotypes between any pair of populations. Nevertheless, results from chi-squared analyses indicated that differences among haplotype frequencies were highly significant ($P = 0.001$). Haplotype '1' occurred in all populations and was the most common haplotype in each population, found in 91.7% of mussels in the North Fork Holston, 41.7% of the Middle Fork Holston, 33.3% of the Clinch, 90% of Paint Rock and 35.7% of the Duck River mussels. Of haplotypes occurring at lower frequencies, the Duck River population had the highest number of unique haplotypes (Table 2). Results from AMOVA showed that 88.3% of variation for *ITS-1* in *L. dolabelloides* resided within populations, with 11.7% among populations.

Results from F_{ST} provided limited support for the inference that the Duck River population was comparatively distinct from the remaining populations. Significant values ($P = 0.017-0.020$) were found between the Duck River population and those from the North Fork Holston and Paint Rock rivers. There also was significant differentiation ($P = 0.010-0.046$) among some pair-wise combinations of the four populations from the middle and upper Tennessee River (Table 3).

Numbers of nucleotide substitutions (J-C) within populations ranged from 0 to 0.866% and averaged 0.499% (Table 3). Corrected J-C values between populations ranged from 0 to 0.275% and averaged 0.114%. Correlation of J-C values and stream distance (Mantel test) showed that the relationship ($r = -0.602$) was not significant ($P = 0.905$), suggesting no pattern of isolation-by-distance.

The minimum-spanning network from *ITS-1* data did not follow population boundaries (Fig. 2B). All haplotypes scored could be linked by three or fewer mutational steps to haplotype '1' ('1NMCPCD'), which was exhibited by 59% of the *L. dolabelloides* individuals sequenced.

Phylogenetic analysis under MP yielded a most parsimonious tree with a total length of 70 steps and CI of 0.971. Only two branches in the final phylogram were supported by >50% bootstrap support (Fig. 4), and none of the populations were monophyletic compared with the remaining populations.

For gene diversity within populations, results from *ITS-1* showed the greatest nucleotide diversity (0.34%) in the Clinch River population (Table 2), with lower values for the Middle Fork Holston and Duck River mussels (0.25–0.32%) and least (0.08%) in the Paint Rock and North Fork Holston River mussels.

DISCUSSION

The study of geographic differentiation in species contributes to understanding current and historic patterns of genetic diversity, and to conserving the processes that shape these patterns. Therefore, our discussion of the current distribution of genetic variation among populations of *L. dolabelloides* will focus on inferred historical patterns of distribution and diversity, and present implications for future translocation or re-stocking programmes for this species.

Genetic structure

Our results suggested substantial geographic genetic structuring in *L. dolabelloides*. Significant haplotype frequency differences for both *ND-1* and *ITS-1* DNA sequences were found among populations. Furthermore, significant among-population (i.e. among-drainage) AMOVA values were estimated for *ND-1* (0.171) and *ITS-1* (0.117). The latter values are closely comparable to the mtDNA-based AMOVA value of 0.118 estimated for populations of *Lampsilis hydiae* in different river drainages by Turner *et al.* (2000). These authors described their value as indicative of significant structuring, consistent with the cessation of gene flow resulting from allopatric fragmentation. In our study, pairwise analysis of F_{ST} values from *ND-1* showed that the most significant divergence was between the Duck River population, the Paint Rock River population and the remaining three populations. The distinctiveness of the Paint Rock population probably reflects loss of genetic diversity rather than real differentiation, since only one haplotype was observed in this population, and this haplotype is shared with three other populations. By contrast, the apparent distinctiveness of the Duck River population reflects the presence of numerous unique haplotypes, suggesting that differentiation between this population, and *L. dolabelloides* from the middle and upper Tennessee River drainage is real.

The F_{ST} values from *ITS-1* supported inference of a measure of uniqueness in the Duck River population and revealed further structuring among populations, with significant F_{ST} values between several pairwise combinations of populations in the middle and upper Tennessee River system. All significant pairwise differences involved the Paint Rock River and North Fork Holston River. Both of these populations of *L. dolabelloides* showed loss of genetic diversity based on *ITS-1* derived data; in both instances, there are well-documented cases of

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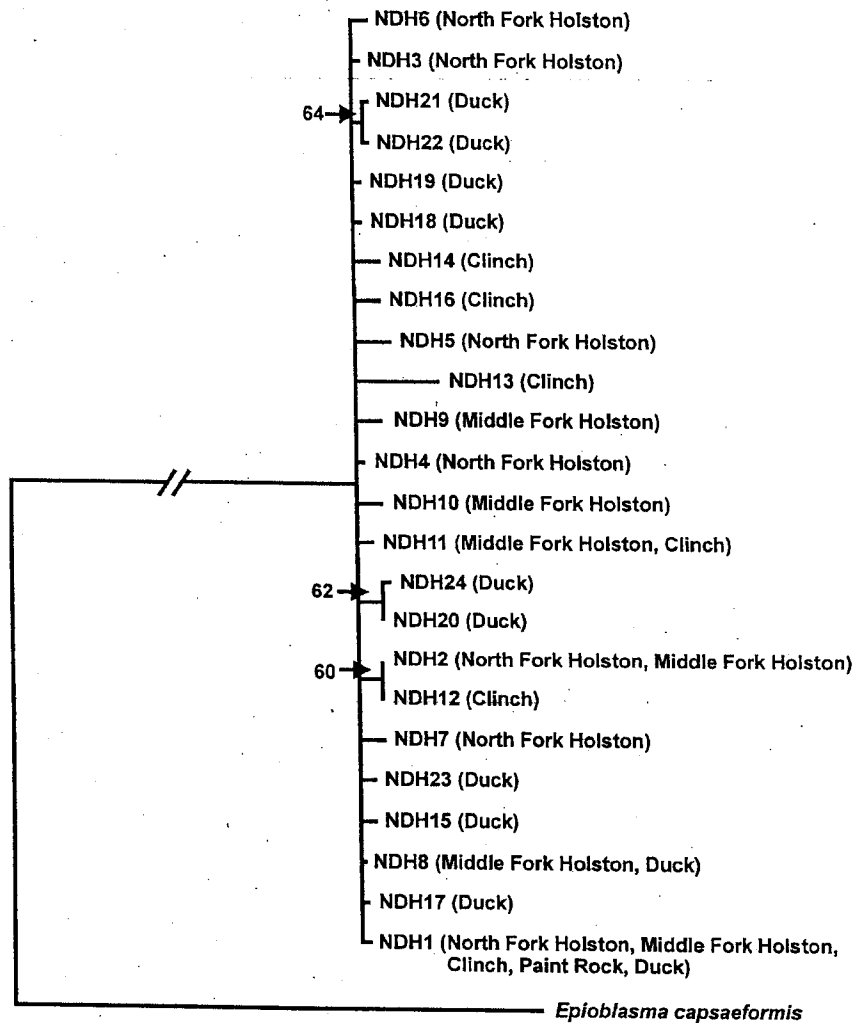


Figure 3. The most parsimonious phylogram resolved for five populations of *Lexingtonia dolabelloides*, from *ND-1* data. Numbers above branches indicate the percentage of bootstrap replicates where the clade was found. Labels indicate haplotype numbers, with the population(s) where the haplotype occurred in brackets.

anthropogenic events that may have led to population bottleneck events in these populations (Neves & Zale, 1982; Ahlstedt, 1995; Henley & Neves, 1999).

The minimum-spanning network based on *ND-1* sequences (Fig. 2A) provides some support for the hypothesis of genetic structuring in *L. dolabelloides*. Topology of the network is based on two domains: a domain consisting of relatively rare haplotypes from the Clinch, North Fork Holston and Middle Fork Holston rivers, and a largely Duck River-centric domain, which also contains the most common and most widespread haplotype in the remaining populations. This topology is consistent with a hypothesis of historical isolation of the Clinch River, North Fork Holston River and Middle Fork Holston River domain, with a limited influx of haplotypes from the other domain.

Genetic structuring in *L. dolabelloides* was less evident from the number of nucleotide substitutions between populations (drainages). Roe & Lydeard (1998) calculated K2P distances of 1.93–2.62 among forms of *Potamilus inflatus*, and used these values to support their conclusion that the two forms should be recognized as separate species, since these values were higher than the K2P values of 1.22–1.40% calculated between

valid *Potamilus* species. By comparison, the average K2P value calculated from *ND-1* data during the present study was 0.584%, with even lower values from *ITS-1* data (average number of substitutions = 0.114%). We note that results may not be directly comparable, since Roe & Lydeard used a different mtDNA gene region compared with the present study, with a potentially different rate and nature of mutation. Nevertheless, two of the pairwise values calculated for *ND-1* fall in the range of 1.22–1.4% found by Roe & Lydeard (1998) between recognized species in the genus *Potamilus*. These values occurred between the Duck and Clinch River populations, and between the Paint Rock and Clinch River populations, supporting the hypothesis of structuring in *L. dolabelloides*.

There has been considerable debate on the units, terminology and criteria for conservation of geographic genetic variants with varying levels of evolutionary potential (Bowen, 1998; Nammack, 1998). From our phylogenetic analysis, using the reciprocal monophyly criterion of Moritz (2002), there was not enough justification for designating any population sampled in the current study as an ESU. Yet, there were significant differences among haplotype frequencies for both genes sequenced. Vogler & DeSalle (1994) considered a biological

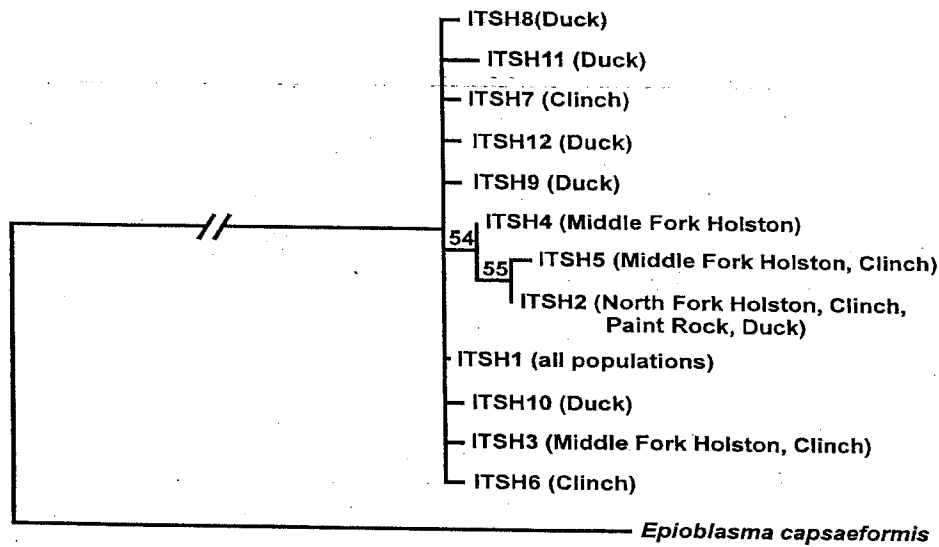


Figure 4. The most parsimonious phylogram resolved for five populations of *Lexingtonia dolabelloides*, from *ITS-1* data. Numbers above branches indicate the percentage of bootstrap replicates where the clade was found. Labels indicate haplotype numbers, with the population(s) where the haplotype occurred in brackets.

unit an ESU only if all individuals in the unit shared at least one heritable trait not found in any individuals from any other units. No populations screened as part of this study met this criterion; there were some unique traits in individuals in specific populations, but never in all individuals from a given population. However, the Duck River population came very close to meeting the criterion. It showed significant differentiation from all other populations at the mitochondrial gene and had several unique haplotypes at the nuclear gene. Hence, a conservative and scientifically defensible option would be to regard populations of *L. dolabelloides* in the Tennessee River system as comprised of two MUs. One MU would comprise the population of *L. dolabelloides* from the Duck River, with the remaining populations in the middle and upper Tennessee River system comprising the second MU. Management units describe the fundamental units of wildlife management (Bowen, 1998), at a category lower than the long-term evolutionary trajectory described by ESU classification. Moritz (2002) defined an MU as having 'significant divergence of allele frequencies at nuclear or mitochondrial loci' and as 'a demographically distinct population that should be managed to ensure the viability of the larger ESU'. In the case of *L. dolabelloides*, the larger ESU is not known, but is probably equivalent to the remaining populations in the Tennessee River drainage.

Moritz (2002) cautioned that molecular criteria impose arbitrary thresholds and categories on an evolutionary process that is in reality based on a continuum of divergence. We also note that these criteria have not been formulated with freshwater mussels as a target faunal group, which may complicate the application of these concepts (Roe & Lydeard, 1998). What is important is to justify why any proposed conservation unit warrants protection. Waples (1991) proposed that ESUs are reproductively isolated from other conspecific units and represent an important component of the evolutionary legacy of the species. To meet the latter criterion, the population must: (1) be genetically distinct, (2) occupy unique habitat, (3) exhibit unique adaptation to its environment, or (4) pose a significant loss to the ecological or genetic diversity of the species if it became extinct. The Duck River population is

geographically isolated from other conspecific populations, but we have no reason to believe that it is reproductively incompatible with the other populations. Nor are we aware of obvious life history, morphological or habitat-related traits for the Duck River population. Therefore, from the available data, none of the *L. dolabelloides* populations sampled, including the Duck, fully meets Waples' (1991) criteria to support classification as an ESU. However, the Duck River population may represent an important component in the evolutionary legacy of the species in that if the population became extinct, it would represent a significant loss of irreplaceable molecular genetic variation for the species as a whole. Management unit status would therefore ensure the conservation of any unique genetic potential in *L. dolabelloides* from the Duck River.

We note that there can be negative consequences from erroneously designating a population unit as an ESU. Such an error could promote gene pool fragmentation and loss through attrition of larger *real* significant units. Erroneous assignment of populations as ESUs also ignores metapopulation structure and does not adequately consider population viability. Roe & Lydeard (1998) suggested that incorrect application of the ESU concept could hinder rather than aid in the recognition of invertebrate biodiversity.

Conservation implications

Recovery plans for many imperiled mussel species include relocation of individuals from demographically robust populations to demographically imperiled ones. The impact of evolutionary and ecological factors on relocation programs must be carefully scrutinized (Villemela *et al.*, 1998). Genetic structuring may be linked to significant adaptive differentiation. If so, relocation without regard to locally adapted genetic factors may result in reduced fitness in progeny. In addition, re-stocking programmes should incorporate the requirement for sufficient genetic diversity in founder populations. The need for genetic diversity is well documented, and relocated populations with low diversity may have reduced ability to thrive, or survive stochastic

perturbations (Villemela *et al.*, 1998). Use of *L. dolabelloides* from the North Fork Holston and Paint Rock Rivers for reintroductions, therefore, should be done only after consideration of the apparent loss of genetic diversity. These populations could, however, be used to maintain overall genetic diversity in the species as part of a metapopulation-based approach. Finally, the availability of the host fish species and habitat quality of the new environment must be considered.

Separate management status for the Duck River raises a challenge for future restocking programmes for *L. dolabelloides*, in addition to the necessity of conserving the Duck River population in its own right. It is difficult to determine whether the genetic uniqueness of the Duck River population reflects historical processes or recent post-impoundment fragmentation. Liu, Herschler & Clift (2003) suggested that lack of contemporary gene flow among hydrographically separated populations might result in pronounced geographical structuring in freshwater molluscs. Thirty-seven dams fragment the Tennessee River drainage, and all five populations surveyed during the current study are isolated from one other by impoundments. Impoundments not only have fragmented the distribution of the species, but also led to a considerable reduction in overall habitat. The species requires suitable substrate in moderately flowing, well-oxygenated waters in order to maintain viable populations (USFWS, 1999). Dams influence breeding of the species both upstream and downstream. *Lexingtonia dolabelloides* will not reproduce under reservoir conditions, and releases of water can degrade large stretches of downstream habitat, rendering it unsuitable for survival. Impoundments can thereby affect freshwater mussels in various ways, and even if man-made barriers to gene flow are not the main cause of genetic fragmentation in *L. dolabelloides*, their influence could certainly compound the effects of historical bottlenecks.

The main argument against an anthropogenic mechanism for genetic uniqueness in the Duck River is the lifespan of freshwater mussels. Man-made barriers to gene flow have existed for less than a century, and the Duck River has been impounded only since 1976 (USFWS, 1999). The maximum age for *L. dolabelloides* has been estimated as at least 40 years, and values up to 100 years have been suggested for other freshwater mussel species in the USA. It thus seems unlikely that fragmentation for a few decades could result in significant genetic divergence unless fragmentation was accompanied by near extirpation, resulting in a pronounced bottleneck. The Duck River contains an isolated but healthy population of *L. dolabelloides*; it thus seems unlikely that substantial molecular differentiation has developed as a result of human influence on gene flow. Therefore, the data seemingly support a hypothesis of historical fragmentation in *L. dolabelloides*, going back before European colonization.

Our results suggest that the pattern of differentiation among populations does not reflect a simple pattern of isolation-by-distance, which also lends support to the hypothesis of historical fragmentation. By contrast, Berg *et al.* (1998) found high levels of among-population gene flow and a pattern of isolation-by-distance in *Quadrula quadrula*. Historical fragmentation in *L. dolabelloides* could be attributed to the habitat specificity of the species, and to complex host fish requirements needed for dispersal and population viability. Gene flow among unionid populations over any significant distance is a function of host fish movements (Berg *et al.*, 1998). Dispersal of *L. dolabelloides* is undescribed in the published literature, but it is probably through transport by various fish species, followed by successful metamorphosis to the juvenile stage and excystment into suitable habitat. Therefore, it is likely that the species has always been fragmented to some extent by geographical separation of suitable habitat, and that this fragmentation has been compounded by human activities. Genetic differentiation among populations would

then result from differing selective influences within the considerable area covered by the Tennessee River drainage, as well as genetic drift.

Historical patterns of differentiation within *L. dolabelloides* would strengthen the argument for recognition of a measure of population distinctiveness in the Duck River population. However, our data provide no reason to restrict reciprocal exchanges between *L. dolabelloides* populations from the North Fork Holston, Middle Fork Holston, Clinch and Paint Rock Rivers, should any of these populations become candidate sources or recipient populations for reintroductions. After considering (i) potential uniqueness of the Duck River population, (ii) stability of the population and (iii) no evident loss of genetic diversity, we recommend that the Duck River population be managed as a separate management unit within the metapopulation of *L. dolabelloides* in the Tennessee River drainage.

In addition to management of the five populations studied, conservation efforts for *L. dolabelloides* will require augmentation of remnant populations and re-introductions to historical rivers, depending on stream habitat restoration and water quality at proposed sites. In the upper Tennessee River system, Neves & Zale (1982) confirmed the presence of *L. dolabelloides* in Big Moccasin Creek (Virginia), a tributary of the North Fork Holston River near the confluence of the North and South forks of the Holston River (Fig. 1). This tributary population could be augmented using individuals from the parent river or from the Middle Fork Holston River. Historical records of *L. dolabelloides* have been reported for the South Fork Holston River (Parmalee & Polhemius, 2004); but the South Holston Dam and impoundment have destroyed primary habitat; therefore, re-introductions are not feasible in this river.

The mainstream of the Holston River historically was inhabited by *L. dolabelloides*, but the species was extirpated from the river by various anthropogenic factors (Ahlstedt, 1991b). Although most of the lower Holston River mainstream is unsuitable mussel habitat due to low water temperature (resulting from hypolimnetic water releases from Cherokee Dam), the upper reaches of the river contain excellent habitat, which could be re-populated using individuals from the North and Middle Forks of the Holston River. Further downstream in the upper Tennessee River, the species historically occurred in the French Broad River (USFWS, 1999), which contains suitable habitat below Douglas Dam; individuals from the more stable populations in the North and Middle forks of the Holston River could in time be translocated to this river and its tributary, the Nolichucky.

A remnant population of *L. dolabelloides* occurs in the Powell River (Parmalee & Bogan, 1998). This population could be augmented using individuals from the population in the Clinch River, or even from the larger population in the Middle Fork Holston River, considering that *L. dolabelloides* in this geographical area constitutes a single management unit. The species also occurred historically in the South Fork Powell in the headwaters of the Powell River, but poor water quality has eliminated suitable habitat (Dennis, 1981). In the remainder of the upper Tennessee River drainage, a remnant population is found in the Hiwassee River (Parmalee & Hughes, 1994). Our genetic data provide no motivation to restrict augmentation of *L. dolabelloides* in this river using individuals from either population in the upper Tennessee River drainage or from the Paint Rock River. However, considering that individuals from the Paint Rock showed some evidence of loss of genetic diversity and differentiation from other populations based on F_{ST} values, using individuals from the upper Tennessee may be more appropriate. Historical habitats in the Little Tennessee, Tellico and Little Pigeon rivers (Parmalee, 1988) in the upper Tennessee River system could similarly be re-established through

introductions from the populations of the North and Middle forks of the Holston River.

In the middle Tennessee River system, remnant populations of *L. dolabelloides* are present in the Elk River (Ahlstedt, 1983; Parmalee & Bogan, 1998) and Bear Creek (McGregor & Garner, 2004). These populations occur between the Paint Rock River population, which is the most unique within the middle and upper Tennessee MU, and the Duck River MU. Consequently, identifying the most appropriate stock for augmentation is problematic. Augmentation of these populations should preferably be delayed until specimens of *L. dolabelloides* from Bear Creek and Elk River are sampled and genetically screened, but the small size and imperiled status of these populations make collection of adequate specimens unlikely. However, management units are primarily concerned with short-term management issues; i.e. MU status prescribes preferential source and recipient populations, but does not exclude reciprocal exchanges between different units absolutely. Augmentation of the Elk River and Bear Creek populations could thus continue from any available source population, if mandated by further population decline. The population that historically occurred in the Buffalo River (Parmalee & Bogan, 1998) was most likely genetically similar to that in the Duck River; thus, this river and its tributary could be augmented using parent stock from the Duck River.

In the Cumberland River System (Fig. 1), *L. dolabelloides* historically occurred in the Red River and Caney Fork River (Layzer et al., 1993), and probably along most of the mainstem downstream from Caney Fork (Parmalee et al., 1980; Starnes & Bogan, 1988; Gordon & Layzer, 1989; USFWS, 1999). Since the species was always rare in the Cumberland River System and habitats have been destroyed, re-introductions are unlikely. Should re-introductions into this system become viable, a clear interpretation of the boundary for the Duck River MU group would be required, in order to decide whether mussels for re-introduction should be sourced in the Duck or from further upstream in the Tennessee River drainage.

Conclusions

The results of our study identify two MUs in *L. dolabelloides* in the Tennessee River drainage. We recommend collection of additional data to assess population genetic fragmentation and appropriate units for conservation, most notably by investigating potentially unique life history traits, morphological characteristics or habitat use, since multiple data sets can provide much needed insight into evolutionary processes (Mulvey et al., 1997; Roe & Lydeard, 1998). In addition, it would be worthwhile to compare the current results to findings from more rapidly-evolving nuclear markers such as microsatellites. Finally, positive identification of host fish species and data on the biogeography of this species is necessary since, ultimately, dispersal patterns in freshwater mussels largely reflect the dispersal ability of their fish hosts.

ACKNOWLEDGEMENTS

The authors acknowledge financial support from The Nature Conservancy and the U. S. Fish and Wildlife Service. We thank Jeff Garner, Alabama Department of Fish and Game, and Paul Johnson, Tennessee Aquarium Research Institute, for their assistance with obtaining mussel tissue samples from the Paint Rock River, AL.

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