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Best regards, Randy

Intraspecific phylogeography of *Lasmigona subviridis* (Bivalvia: Unionidae): conservation implications of range discontinuity

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Abstract

A nucleotide sequence analysis of the first internal transcribed spacer region (ITS-1) between the 5.8S and 18S ribosomal DNA genes (640 bp) and cytochrome *c* oxidase subunit I (COI) of mitochondrial DNA (mtDNA) (576 bp) was conducted for the freshwater bivalve *Lasmigona subviridis* and three congeners to determine the utility of these regions in identifying phylogeographic and phylogenetic structure. Sequence analysis of the ITS-1 region indicated a zone of discontinuity in the genetic population structure between a group of *L. subviridis* populations inhabiting the Susquehanna and Potomac Rivers and more southern populations. Moreover, haplotype patterns resulting from variation in the COI region suggested an absence of gene exchange between tributaries within two different river drainages, as well as between adjacent rivers systems. The authors recommend that the northern and southern populations, which are reproductively isolated and constitute evolutionarily significant lineages, be managed as separate conservation units. Results from the COI region suggest that, in some cases, unionid relocations should be avoided between tributaries of the same drainage because these populations may have been reproductively isolated for thousands of generations. Therefore, unionid bivalves distributed among discontinuous habitats (e.g. Atlantic slope drainages) potentially should be considered evolutionarily distinct. The DNA sequence divergences observed in the nuclear and mtDNA regions among the *Lasmigona* species were congruent, although the level of divergence in the COI region was up to three times greater. The genus *Lasmigona*, as represented by the four species surveyed in this study, may not be monophyletic.

Keywords: COI, ITS-1, *Lasmigona subviridis*, phylogenetics, phylogeography

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Introduction

Due to unprecedented levels of anthropogenic elimination and/or degradation of contiguous habitats, aquatic species diversity is increasingly threatened (Erwin 1991). Perhaps no faunal group illustrates this trend more aptly than freshwater bivalves of the family Unionidae. North America possesses the world's greatest diversity of freshwater bivalves and more than 70% of recognized species are considered to be endangered, threatened, or of special concern (Williams *et al.* 1993). Primary threats to these bivalves and their habitat are pollution, increased sedimentation resulting from stream alterations, loss of host fish species that sustain the parasitic unionid larvae (glochidia) during early development, and the aggressive expansion of the exotic zebra mussel (*Dreissena polymorpha*) (Williams *et al.* 1993). An integrative conservation approach that identifies and sustains ecological processes and evolutionary lineages is urgently needed to protect and manage freshwater bivalve biodiversity. However, these bivalves present significant challenges to conservation biologists in the form of broad taxonomic uncertainty due to their phenotypic plasticity, complex life histories, and varied modes of reproduction.

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Effective management of at-risk species can be complicated because adequate taxonomic information for identification of species or distinct intraspecific populations may not exist. Inferred phylogeny of unionids is customarily drawn from comparative morphology of the modern fauna (Heard & Guckert 1971; Taylor 1988) which is characterized by a high degree of phenotypic plasticity in conchology and soft-part morphology (Kat 1983a). A morphology-based taxonomy may not reveal true phylogenetic relationships as the rate of evolutionary change can vary among lineages and similar environmental influences may cause convergence (Grant 1987). Therefore, a unionid bivalve taxonomy based solely on phenotypic characteristics can complicate conservation efforts (Williams & Mulvey 1994; Lydeard & Roe 1998) and ultimately jeopardize a species' ecological and evolutionary potential. In contrast, molecular data have a clear heritable genetic basis with the number of characters limited only by genome size (Moritz & Hillis 1996). Moreover, molecular genetic markers can quantify the extent of reproductive relationships and may more accurately reflect true evolutionary relationships (i.e. phylogeny), including divergence (Avise 1994).

Coupled with uncertainties in taxonomy, many unionid bivalves possess a complex life cycle in which they are relatively sedentary as adults, relying on temporary attachment of glochidia to a mobile host (usually a fish) for dispersal. Consistent with this dependence, a positive correlation has been observed between unionid bivalve diversity and fish diversity (Watters 1992). A relationship may also exist between bivalve and fish distribution such that gene flow among populations of unionids is dependent upon the parasitized host. Therefore, the vagility of the parasitized host can have a profound effect on the levels of gene flow and ultimately the rate of divergence among populations (Kat 1984). The hosts remain unknown for the vast majority of unionid species, further inhibiting conservation efforts.

Observed patterns in genetic variation have been shown to correlate highly with historical biogeographic factors (Bermingham & Moritz 1998, and references contained within that issue). The geographical distribution of unionid lineages may be used to infer (or confirm) the history of a bivalve and host species' expansion and the presence (or cessation) of migration among extant populations by the host (Bermingham & Martin 1998). In the absence of fundamental knowledge concerning a unionid bivalve's host(s), information on bivalve population genetic structure combined with historical biogeographic data could be used to deduce intraspecific phylogeographic structure. This structure could then be used to develop enlightened strategies for the conservation of evolutionarily distinct bivalve lineages.

Few studies comparing intraspecific phylogeography have been conducted on unionid bivalves (but see Lydeard *et al.* 1996; Mulvey *et al.* 1997; Roe & Lydeard 1998). These studies, which analysed DNA sequence information, have shown that molecular genetic data can offer a robust tool for deducing unionid bivalve phylogeographic structure and have questioned the validity of phylogenetic hypotheses generated by conventional morphological analyses. For example, Lydeard *et al.* 1996 demonstrated that many of the morphological traits that have served as the basis for the contemporary classification scheme of unionids were found to be homoplastic when mapped on a DNA-based phylogeny. Ultimately, the molecular genetic data generated in each of the studies augmented conservation efforts directed at the taxa in question.

The green floater *Lasmigona subviridis* (Bivalvia: Unionidae) is a freshwater bivalve identified as a species in need of acute conservation efforts. Historically, the distribution of *L. subviridis* included the US Atlantic slope from the small tributaries of the Hudson River drainage, New York to the Cape Fear river system in North Carolina, as well as the Kanawha–New system in the Ohio–Mississippi river drainage where it is thought to have originated (Clarke 1985). Currently, a few disjunct populations exist in small, headwater streams. Information on *L. subviridis* population structure and general habitat requirements is lacking. Moreover, the host species is unknown for *L. subviridis*, which has been shown to be a simultaneous hermaphrodite (Clarke 1985). *L. subviridis* is under consideration for petition to be protected under the Endangered Species Act as part of a management strategy to preserve and restore the historical distribution. A thorough understanding of the phylogeographic relationships among populations of *L. subviridis* is essential to provide information needed to assess the species' status and for planning and implementing biologically sound management programmes. Towards that end, the objectives of the present study were to assess the utility of DNA sequence variation for detecting phylogeographic structure among *L. subviridis* populations and to examine the implications of the findings in light of conservation priorities.

To characterize the intraspecific phylogeographic structure of *L. subviridis* populations, we used nucleotide sequence analysis of the internal transcribed spacer region (ITS-1) between 5.8S and 18S ribosomal DNA genes and the first subunit of the cytochrome *c* oxidase (COI) region of mitochondrial DNA (mtDNA). We believe this study represents the first investigation into the population genetic structure of *L. subviridis* and the first survey of sequence variation at ITS-1 among geographically distant populations of any freshwater bivalve. DNA sequences from representatives of *Lasmigona compressa*, *Lasmigona complanata*, and *Lasmigona costata* were also analysed to facilitate comparison of differentiation observed among *L. subviridis* populations with that observed among well-established congeneric species.

Table 1 Major river drainages, tributary or general collection localities, and sample sizes for *Lasmigona* and outgroup specimens sequenced for the internal transcribed spacer region (ITS-1) between 18S and 5.8S and the first subunit of cytochrome *c* oxidase (COI) gene of mitochondrial DNA. Specific collection localities can be obtained from the corresponding author

Species	Drainage	Tributary/Location	Locality	Sample Size	
				COI	ITS-1
Lasmigona subviridis	Susquehanna River	Pine Creek, PA	1	5	6
		Sidling Hill Creek, PA	2	7	9
	Potomac River	Sidling Hill Creek, MD	3	1	1
		Cacapon River, WV	4	5	5
	Rappahanock River	Fauqueir County, VA	5	2	1
	James River	Rivanna River, VA	6	2	2
		Tye River, VA	7	6	5
	Neuse River	Little River, NC	8	5	5
	Kanawha River	Greenbrier River, WV	9	4	6
L. complanata complanata	Mississippi River	Victory, WI		2	2
L. complanata alabamensis	Coosa River	Cherokee County, AL		1	—
L. compressa	Mississippi River	Jordan Creek, IL		2	2
L. costata	Poultney River	West Haven, VT		2	2
Alasmidonta heterodon-1	Ashuelot River	Cheshire County, NH		1	—
A. heterodon-2	Neversink River	Orange County, NY		1	—
A. heterodon-3	Tar River	Granville County, NC		1	1
Strophitus undulatus-1	Connecticut River	Coos County, NH		1	—
S. undulatus-2	Susquehanna River	Pine Creek, PA		1	1
Pyganodon grandis	Cedar River	Gladwin County, MI		1	—
P. fragilis	Freshwater Pond	Burin, Newfoundland, Canada		1	—

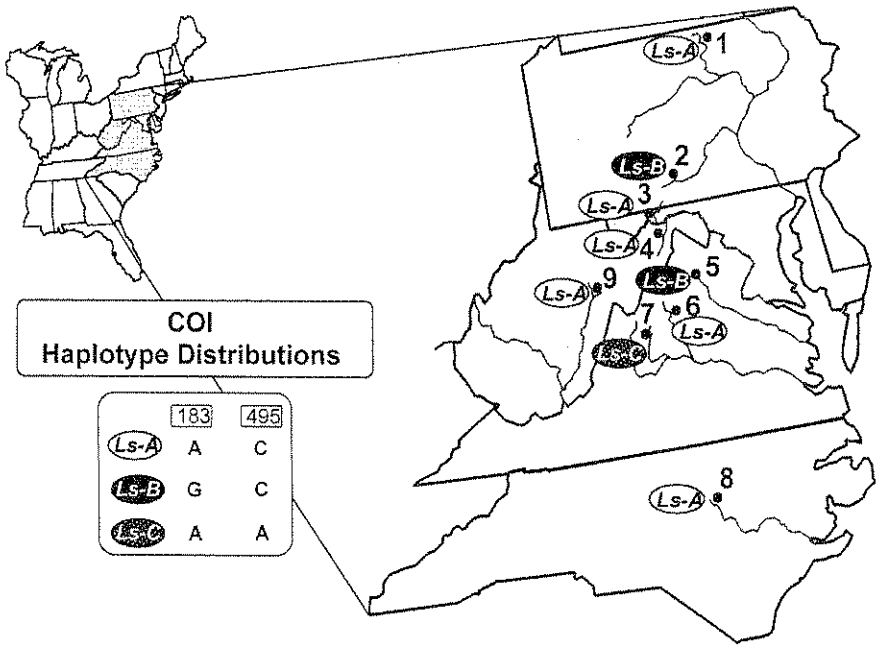

Fig. 1 Collection locations and the distribution of three haplotypes (Ls-A, Ls-B, and Ls-C) generated from sequence variation at the first subunit of the cytochrome *c* oxidase (COI) region of mtDNA in the unionid bivalve *Lasmigona subviridis* collected from nine geographical populations. Locality designations are provided in Table 1.

Materials and methods

Samples and DNA extraction

Lasmigona subviridis samples were collected from nine localities on six river systems (Table 1, Fig. 1). Live *L. subviridis* were shipped to the Leetown Science Center, Kearneysville, WV, USA, where they were maintained in aquaria until mantle or foot tissue was removed and preserved in 95% ethanol prior to DNA extraction and

sequence analysis. Representative specimens of *L. complanata*, *L. compressa*, and *L. costata* were provided as museum specimens preserved in 70% or 95% ethanol (see Table 1).

Total genomic DNA was isolated from approximately 20 mg of mantle or foot tissue using the Puregene DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN, USA) and resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Gonadal tissues were specifically avoided to prevent comparisons of nonorthologous sequences due to the actual or potential presence of doubly uniparental inheritance of mtDNA in some bivalve taxa (e.g. see Skibinski *et al.* 1994; Zouros *et al.* 1994). DNA concentrations were determined by fluorescence assay (Labarca & Paigen 1980) and integrity of the DNA was visually inspected on 1% agarose gels (Sambrook *et al.* 1989).

The same individuals were used to survey sequence variation in both DNA regions, but some individuals amplified at only one of the two regions even after multiple attempts. Therefore, the sample sizes for the two regions differed in some instances.

DNA sequence production

COI. A 710-bp fragment of the cytochrome *c* oxidase subunit I (COI) of mtDNA was amplified from genomic DNA using the PCR. The amplification primers, designed by Folmer *et al.* (1994), were: COI-H, 5′-TAAACTTC-AGGGTGACCAAAAAATCA-3′ and COI-L, 5′-GGTCAA-CAAATCATAAAGATATTGG-3′.

Amplification reactions consisted of 100 ng of genomic DNA, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM each primer, and 1.0 U AmpliTaq® DNA polymerase (Perkin-Elmer Applied Biosystems (ABI), Perkin-Elmer Corp.; Foster City, CA, USA) in a total volume of 20 μL. Amplifications were carried out on a PTC-200 Thermal Cycler (MJ Research) using these conditions: initial denaturing at 94 °C for 2 min; 35 cycles of 94 °C denaturing for 30 s, 54 °C annealing for 30 s, 72 °C extension for 90 s and a final extension at 72 °C for 5 min. The purified COI amplicon was sequenced directly using the amplification primers.

ITS-1. Sequence analysis was performed on 640 bp of the internal transcribed spacer region (ITS-1) separating 5.8S and 18S ribosomal DNA genes amplified by the polymerase chain reaction (PCR). Dr R. Phillips (University of Wisconsin-Milwaukee) designed the amplification primer sequences from the conserved 18S and 5.8S ribosomal DNA genes flanking the spacer region in salmonids. The primers were: ITS-1 18S, 5′-AAAAAG-CTTCCGTAGGTGAACCTGCG-3′ and ITS-1 5.8S, 5′-AGC-TTGCTGCGTTCTTCATCG-3′. PCR reaction components, thermal cycling conditions, and amplicon purification were similar to that described for COI with the exception that the annealing temperature was 64 °C. Amplified PCR products were purified and desalted using Microcon-30 microconcentrators, according to the manufacturer's instructions (Amicon, Beverly, MA, USA). The purified ITS-1 fragments were cloned into the pGEM-T Easy vector system (Promega Corporation, Minneapolis, MN, USA) and sequenced using standard M13 forward and reverse primers (Life Technologies Inc., Rockville, MD, USA). The number of clones of each individual sequenced varied among individuals (from 5 to 10).

Sequence reactions were performed using the ABI Prism Big Dye Terminator Cycle Sequencing reaction kit utilizing AmpliTaq DNA Polymerase, FS (ABI). Cycle sequencing reactions were purified by standard ethanol/sodium acetate precipitation (Sambrook *et al.* 1989). Each purified sample was resuspended in 12–14 μL of template suppression reagent (ABI), denatured at 95 °C for 3 min, chilled on ice for 2 min, and vortexed briefly. Capillary electrophoresis was performed on 12 μL of each sample using the ABI Prism-310 Genetic Analyser and DNA Sequencing Analysis Software (ABI).

Data analyis

Sequence alignment was performed using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Assessment of phylogenetic relationships based on amino acid information content for COI sequences was accomplished by translating codons according to the *Drosophila* mitochondrial code. Based on the results of prior phylogenetic analyses of anodontine bivalves (Hoeh 1990 and Lydeard *et al.* 1996), specimens of *Pyganodon grandis*, *P. fragilis*, *Alasmidonta heterodon* and *Strophitus undulatus* were used to root the resulting COI topology. The phylogenetic relationships from homologous sequences among populations of *L. subviridis*, other *Lasmigona* species, and outgroup species for COI and ITS-1 regions were estimated using the maximum parsimony (MP) and neighbour-joining (NJ) algorithms (PAUP 4.0b; Swofford 1998). The NJ algorithm used the Tamura-Nei distance measure (Tamura & Nei 1993) with rates assumed to follow the gamma distribution with shape parameter = 0.5. Based on the results of the COI analyses, *L. complanata* and *L. costata* were used as outgroups in the ITS-1 analyses. Gaps in the ITS-1 sequence matrix were distributed proportionally to unambiguous changes in the NJ analyses. The robustness of the resulting topologies was tested using bootstrap analyses (1000 replicates for MP and 10 000 replicates for NJ).

The statistical significance of any relationship (i.e. congruence) in sequence differentiation between the ribosomal and mitochondrial DNA regions was determined by sampling the randomization distribution generated from 5000 permutations using the MXCOMP (matrix comparison) routine in NTSYS-PC 1.8 by Rohlf (1993).

Estimates of sequence divergence for the DNA regions were made using the Tamura-Nei model implemented in MEGA (Kumar *et al.* 1993) with indel (insertion and deletion) variation ignored. The MXCOMP routine tested whether the phylogenetic pattern in the two DNA regions is nonrandomly more similar than it would be if there were no common signal. The product–moment correlation, *r*, and the Mantel (1967) test statistic, Z, were calculated to measure the degree of relationship between the two divergence matrices.

Results

COI

Nucleotide sequences of 576 bp in length were obtained from the mitochondrial DNA cytochrome *c* oxidase subunit I (COI) gene for 37 *Lasmigona subviridis* specimens representing nine geographical populations and at least two specimens each of *L. compressa*, *L. complanata*, and *L. costata*. Outgroup taxa consisted of the following: *Alasmidonta heterodon* ($N = 3$), *Strophitus undulatus* ($N = 2$), *Pyganodon grandis* ($N = 1$), and *P. fragilis* ($N = 1$) (Table 1). Analysis of sequence variation at the COI gene among the four *Lasmigona* species revealed 102 phylogenetically informative sites. The transition (Ts) to transversion (Tv) substitution ratio was 5.0 (85/17). Of the 102 informative sites, two were at the first position of the codon, 11 were at the second position, and 89 were at the third. Translation of codons into amino acids indicated 17 variable sites. Within *L. subviridis*, the transversion at site 495, a third position substitution, resulted in an amino acid substitution in the sample from the Tye River, VA. Twenty-two nucleotide substitutions were observed between *L. subviridis* and *L. compressa*; however, only one site (549) resulted in an amino acid substitution. The 15 remaining variable amino acid sites were attributable to the more divergent species, *L. complanata* and *L. costata*.

Among the nine sampling locations for *L. subviridis*, only two variable sites (one transition, one transversion; 0.4% sequence divergence) were observed resulting in three haplotypes (Ls-A, Ls-B, and Ls-C; Fig. 1). No haplotype variation was observed within any locality with the sample sizes available for this rare species. The limited COI sequence variation was geographically informative, as *L. subviridis* inhabiting different tributaries within river systems exhibited distinct mtDNA haplotypes (Fig. 1). *L. subviridis* from Sideling Hill, PA ($N = 7$; locality 2) was found to possess haplotype Ls-B while all specimens from the other Susquehanna River tributary, Pine Creek ($N = 5$; locality 1), exhibited the predominant haplotype, Ls-A. Similarly, for the tributaries of the James River, specimens from the Rivanna River ($N = 6$; locality 6) were all found to possess haplotype Ls-A while both *L. subviridis* from the Tye River (locality 7) were found to have a unique haplotype, Ls-C. *L. subviridis* from the Rappahannock River, VA ($N = 2$; locality 5) were uniquely differentiated from adjacent rivers, constituting the only specimens collected south of the Potomac River exhibiting haplotype Ls-B.

Pairwise comparisons of COI sequence divergence using the Tamura-Nei model for all *Lasmigona* haplotypes, an *A. heterodon* individual, and a *S. undulatus* individual are presented in Table 3. Pairwise per cent sequence divergence between the three *L. subviridis* haplotypes were 0.17% (between Ls-A–Ls-C and Ls-A–Ls-B) and 0.35% (between Ls-B–Ls-C). Within the genus *Lasmigona*, interspecific pairwise sequence differences ranged from 3.81% to 15.03%. Figure 2 represents the single, most parsimonious MP tree obtained from analyses of the COI nucleotide sequences with boostrap percentages for MP (above branches) and NJ (below branches) analyses. The NJ tree (not shown) was congruent with the MP tree. The parsimony and NJ analyses suggest that the three *L. subviridis* COI haplotypes formed a monophyletic group with its sister taxon being *L. compressa*. As it is not possible to root the MP topology such that all *Lasmigona* species form a clade (to the exclusion of the other genera), the genus *Lasmigona* may not constitute a monophyletic group.

ITS-1

Homologous nucleotide sequences were obtained from the first internal transcribed spacer region (ITS-1) between 18S and 5.8S rDNA genes for 46 freshwater bivalves of the genus *Lasmigona* including *L. subviridis* ($N = 40$), *L. complanata* ($N = 2$), *L. compressa* ($N = 2$), *L. costata* ($N = 2$). One reference specimen each of *A. heterodon* and *Strophitus undulatus* was sequenced and compared to the *Lasmigona* species. Due to the repeated nature of this nuclear rDNA array, multiple clones of each individual were sequenced to test for intra-individual variation. Consensus sequences for two *L. subviridis* individuals contained a large number of indels (insertions or deletions) such that the length of amplified fragment varied greatly within the individual. This was assumed to represent variation among array units and these individuals were excluded from further analysis.

The total aligned data matrix for the four *Lasmigona* species, *A. heterodon*, and *S. undulatus*, including indels, was 640 bp. Comparing all *Lasmigona* species, 36 variable sites (5.6%) were identified and 26 (4.1%) of the substitutions were phylogenetically informative. Interspecific sequence comparison resulted in a transversion bias in the Ts/Tv ratio as a value of 0.5 (11/22) was obtained. Indels were observed with most centred in two small simple sequence repeat motifs (or microsatellites). A dinucleotide microsatellite $(GT)_n$ varied from five (*L. subviridis*) to

Table 2 Observed and grouped (major) genotypes resulting from the variable sites matrix (excluding insertions and deletions) of the first internal transcribed spacer (ITS-1) region between 18S and 5.8S ribosomal DNA genes (641 bp) observed in *Lasmigona subviridis* and three congeneric species. Representative sequences for major genotypes Ls-1 and Ls-2 in *L. subviridis* have been submitted to GenBank under Accession nos AF093838 and AF091331, respectively

		Site				
Observed genotype	Animal number	11 5666779911 7239157812	1111112333 3667795035 9172306431	3333444444 5678013446 4240433562	444456 888903 689126	Major genotype
L. subviridis-1	Lsu1A	ATGATTACGC	CACTAGGCAT	C-AA-ATCCA	ACTTGC	Ls-1
L. subviridis-1	Lsu1B	....	....	....	......	Ls-1
L. subviridis-2	Lsu1C	....	....	....	.....A	Ls-1
L. subviridis-2	Lsu1D	....	....	....	.....A	Ls-1
L. subviridis-2	Lsu1E	....	....	....	.....A	Ls-1
L. subviridis-2	Lsu1F	....	....	....	.....A	Ls-1
L. subviridis-1	Lsu2A	....	....	....	......	Ls-1
L. subviridis-2	Lsu2B	....	....	....	.....A	Ls-1
L. subviridis-2	Lsu2C	....	....	....	.....A	Ls-1
L. subviridis-1	Lsu2D	....	....	....	......	Ls-1
L. subviridis-1	Lsu2E	....	....	....	......	Ls-1
L. subviridis-1	Lsu2F	....	....	....	......	Ls-1
L. subviridis-1	Lsu2G	....	....	....	......	Ls-1
L. subviridis-2	Lsu2H	....	....	....	.....A	Ls-1
L. subviridis-1	Lsu2I	....	....	....	......	Ls-1
L. subviridis-1	Lsu3A	....	....	....	......	Ls-1
L. subviridis-1	Lsu4A	....	....	....	......	Ls-1
L. subviridis-2	Lsu4B	....	....	....	.....A	Ls-1
L. subviridis-3	Lsu4C	....	....	....	....AA	Ls-1
L. subviridis-2	Lsu4D	....	....	....	.....A	Ls-1
L. subviridis-1	Lsu4E	....	....	....	......	Ls-1
L. subviridis-4	Lsu5A	C.........	A.........	.......G.	.....A	Ls-2
L. subviridis-5	Lsu6A	C.........	A.........	.......G.	......	Ls-2
L. subviridis-4	Lsu6B	C.........	A.........	.......G.	.....A	Ls-2
L. subviridis-4	Lsu6C	C.........	A.........	.......G.	.....A	Ls-2
L. subviridis-5	Lsu6D	C.........	A.........	.......G.	......	Ls-2
L. subviridis-5	Lsu6E	C.........	A.........	.......G.	......	Ls-2
L. subviridis-4	Lsu7A	C.........	A.........	.......G.	.....A	Ls-2
L. subviridis-5	Lsu7B	C.........	A.........	.......G.	......	Ls-2
L. subviridis-5	Lsu8A	C.........	A.........	.......G.	......	Ls-2
L. subviridis-5	Lsu8B	C.........	A.........	.......G.	......	Ls-2
L. subviridis-5	Lsu8C	C.........	A.........	.......G.	......	Ls-2
L. subviridis-4	Lsu8D	C.........	A.........	.......G.	.....A	Ls-2
L. subviridis-5	Lsu8E	C.........	A.........	.......G.	......	Ls-2
L. subviridis-4	Lsu9A	C.........	A.........	.......G.	.....A	Ls-2
L. subviridis-6	Lsu9B	C....G...	A.C......	.......G.	.....A	Ls-2
L. subviridis-5	Lsu9C	C.........	A.........	.......G.	......	Ls-2
L. subviridis-4	Lsu9D	C.........	A.........	.......G.	.....A	Ls-2
L. subviridis-5	Lsu9E	C.........	A.........	.......G.	......	Ls-2
L. subviridis-5	Lsu9F	C.........	A.........	.......G.	......	Ls-2
L. compressa-1		CA.......	A.........	....T.G.	.....A	
L. compressa-2		CA.......	A.........	.......G.	.....A	
L. costata-1		CA.T.-.GAA	ACA.G-.TTC	AGCGGTAGTC	-.-C.A	
L. costata-2		C.ATC-.G--	ACA.G-.TTC	AACGGTAGTC	-.-C.A	
L. complanata-1		C. T.G.G--	ACA..-TTT.	A.CGGT.G..	CTCC.A	
L. complanata-2		C..T.G.G--	ACA..TTTT.	A.CGGT.G..	CTCC.A	

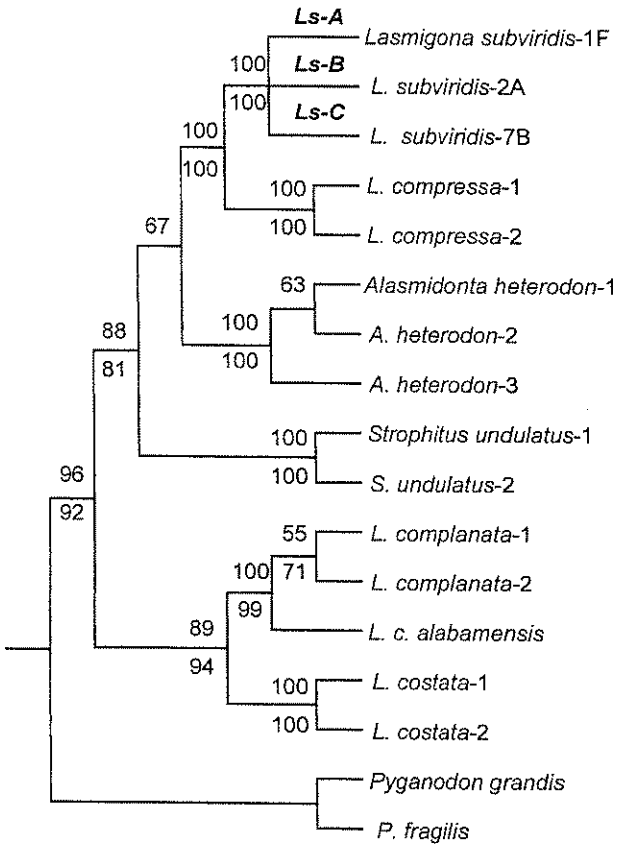

Fig. 2 Best tree topology produced by maximum parsimony analysis of the first subunit of the cytochrome *c* oxidase (COI) nucleotide matrix. Numerals are bootstrap percentages for MP (above branches) and NJ (below branches) analyses. Only bootstrap values greater than 50% are shown. One individual possessing each of the three COI haplotypes is presented in the tree. The corresponding haplotypes (Ls-A, Ls-B, and Ls-C) are provided above the respective branch on the tree.

12 (*L. compressa*) repeat units and was informative in determining phylogeographic structure in *L. subviridis*. A trinucleotide microsatellite $(TAC)_n$ varied from three repeat units in *L. subviridis* and *L. complanata* to seven units in *L. compressa*.

Among nine populations of *L. subviridis*, seven of 640 bp (or 1.1%) were variable, involving substitutions resulting in six observed genotypes. Three substitutions (sites 57, 139, and 445) constituted transversions and were found to be phylogenetically informative. Of the remaining four substitutions, three were unique to two individuals and one (site 636) varied within populations (see Table 2 for observed genotypic assignments). In addition to nucleotide substitutions, four indels consisting of two repeats of the GT dinucleotide microsatellite were observed.

Genotypes consisting of the indel variation combined with the three transversional substitutions were geographically distributed into two major genotypes (Ls-1 and Ls-2; Table 2) such that a discontinuity in genetic population structure between northern and southern *L. subviridis* populations was evident. Diagnostic DNA sequences were observed between the genotype observed in four populations of the Susquehanna and Potomac Rivers (genotype Ls-1; localities 1–4) and the five southern river populations (Ls-2) which included the Rappahannock River, Tye and Rivanna Rivers of the James River drainage, the Greenbrier River, and the Little River (Fig. 3).

Per cent sequence divergence (calculated without indels) between Ls-1 and Ls-2 using the Tamura-Nei model was 0.5%. Among all *Lasmigona* species, pairwise sequence differences ranged between 0.2% (Ls-2 and *L. compressa*) and 5.1% (Ls-1 and *L. costata*) (Table 3, below diagonal). The sequence divergence was greater between Ls-1 and Ls-2 genotypes than between Ls-2 and *L. compressa*, which differed from Ls-2 by one transitional substitution. However, the two major *L. subviridis* genotypes differed from *L. compressa* in possessing differences in the number of repeat units in both microsatellites.

Based on the topologies obtained from analyses of the COI sequences, the ITS-1 sequences were rooted using *L. costata* and *L. complanata* as outgroups. The MP analysis resulted in five equally parsimonious trees and the strict consensus tree with bootstrap estimates is presented in Fig. 4. The NJ bootstrap estimates are also provided (below branches). Graphical depiction of the relationships among the ITS-1 sequences by the parsimony and NJ algorithms, while distinguishing the two major *L. subviridis* genotypes (Ls-1 and Ls-2), indicated that ITS-1 possesses relatively weak phylogenetic signal in discriminating *L. subviridis* and *L. compressa*. The MP and NJ trees suggest that the southern genotype (Ls-2) of *L. subviridis* is the less divergent genotype when compared to *L. compressa* and the other *Lasmigona* species.

Intergenomic comparison

Intraspecific DNA sequence variation in ribosomal DNA (ITS-1) and mitochondrial DNA (COI) regions among geographical populations of *L. subviridis* lacked congruence. The discontinuity identified between northern (localities 1–4; Ls-1) and southern (localities 5–9; Ls-2) populations in the ITS-1 region was not observed in the COI sequences. Similarly, the COI haplotype discontinuities observed between tributaries within the Susquehanna and James Rivers were not mirrored in the ITS-1 sequences.

The DNA sequence divergence observed in the nuclear ribosomal ITS-1 and mtDNA COI regions within and among geographical populations of the four species of *Lasmigona*, *A. heterodon*, and *S. undulatus* was relatively congruent both statistically and graphically (Figs 2 and 4), although the level of divergence detected was up to three-times greater for the COI region. The Mantel test

Table 3 Pairwise genetic distance matrices based on the Tamura-Nei model (Tamura & Nei 1993) generated from 573 bp of the mitochondrial cytochrome oxidase *c* subunit I gene (above the diagonal) and 641 bp of the first internal transcribed spacer region (ITS-1) between 18S and 5.8S rDNA genes (below). Values represent percentage of nucleotide substitutions between sequences and do not reflect observed indels. *Lasmigona subviridis* individuals were chosen to incorporate the three COI haplotypes and the two major ITS-1 genotypes. These matrices were subjected to a Mantel test for test of congruence between the two regions of DNA

	Lsu1F	Lsu2A	Lsu7B	*L. compressa*-1	*L. compressa*-2	*L. costata*-1	*L. costata*-2	*L. complanata*-1	*L. complanata*-2	*Alasmidonta heterodon*-3	*Strophitus undulatus*-2
Lsu1F		0.0017	0.0017	0.0381	0.0381	0.1487	0.1487	0.1209	0.1259	0.1069	0.1168
Lsu2A	0.0018		0.0035	0.0400	0.0400	0.1466	0.1466	0.1229	0.1279	0.1088	0.1189
Lsu7B	0.0074	0.0055		0.0399	0.0399	0.1466	0.1466	0.1189	0.1239	0.1089	0.1188
L. compressa-1	0.0074	0.0092	0.0037		0.0000	0.1503	0.1503	0.1274	0.1275	0.0993	0.1093
L. compressa-2	0.0074	0.0092	0.0037	0.0000		0.1503	0.1503	0.1274	0.1275	0.0993	0.1093
L. costata-1	0.0300	0.0319	0.0261	0.0223	0.0223		0.0000	0.1081	0.1039	0.1504	0.1641
L. costata-2	0.0319	0.0338	0.0280	0.0280	0.0280	0.0055		0.1081	0.1039	0.1504	0.1641
L. complanata-1	0.0243	0.0261	0.0205	0.0205	0.0205	0.0130	0.0148		0.0106	0.1416	0.1405
L. complanata-2	0.0243	0.0261	0.0205	0.0205	0.0205	0.0130	0.0148	0.0000		0.1424	0.1387
A. heterodon-3	0.0319	0.0338	0.0280	0.0280	0.0280	0.0300	0.0319	0.0243	0.0243		0.1196
S. undulatus-2	0.0435	0.0454	0.0435	0.0435	0.0435	0.0513	0.0493	0.0415	0.0415	0.0474	

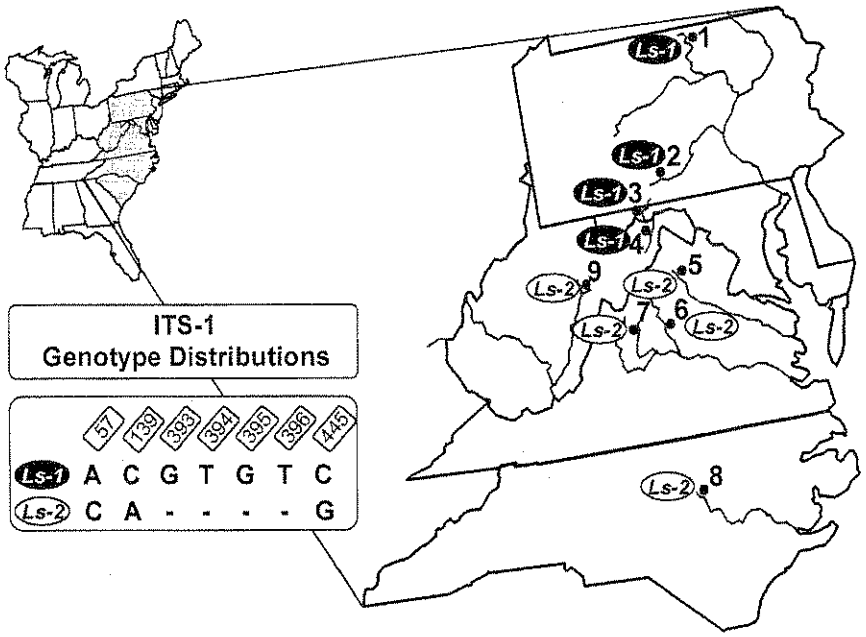

Fig. 3 Collection locations and distribution of the two major genotypes (Ls-1 and Ls-2) generated from sequence variation at the first internal transcribed spacer region (ITS-1) between 18S and 5.8S ribosomal DNA genes in the unionid bivalve *Lasmigona subviridis* collected from nine geographical populations. Locality designations are provided in Table 1.

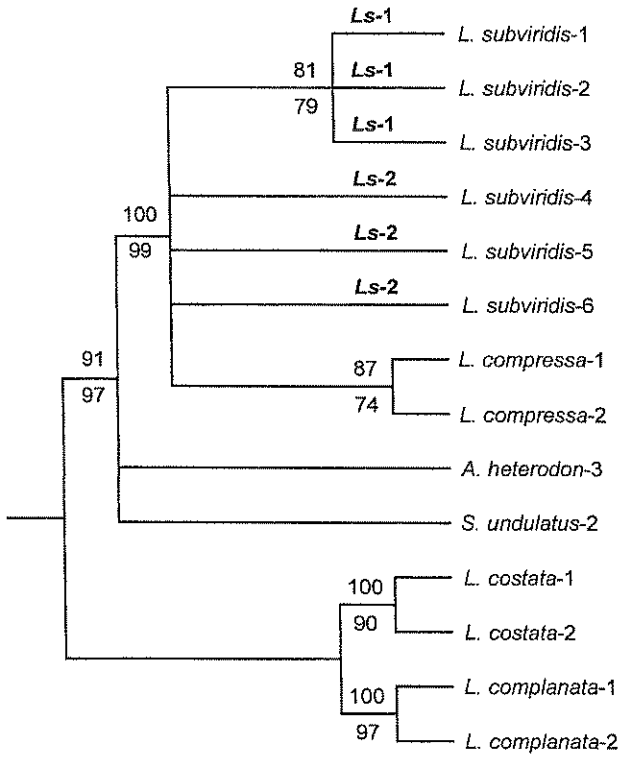

Fig. 4 Best tree topology produced by maximum parsimony analysis of the nucleotide matrix of the first internal transcribed spacer region (ITS-1) between 18S and 5.8S ribosomal DNA genes. Numerals are bootstrap percentages for MP (above branches) and N-J (below branches) analyses. Only bootstrap values greater than 50% are shown. Each of the six ITS-1 genotypes (described in Table 2) is presented in the tree. The corresponding major haplotype (Ls-1 and Ls-2) is provided above the respective branch on the tree.

identified a strong, positive correlation ($r = 0.84$) between the two Tamura-Nei distance matrices (Table 3) and the relationship between these matrices (determined by sampling the randomization distribution generated from 5000 permutations) was found to be statistically significant ($Z = 4.573$; $P < 0.0001$). Graphically, the underlying patterns in the distance matrices depicted similar phylogenetic relationships among the six species (Figs 2 and 4).

Discussion

Phylogeography of Lasmigona subviridis

The present study was conducted to determine the phylogeographic structure among populations of the at-risk unionid bivalve *L. subviridis* using the cytochrome *c* oxidase subunit I (COI) region of mtDNA and a nuclear ribosomal DNA internal transcribed spacer region (ITS-1). To our knowledge, this study represents the first effort to characterize genetic population structure in this species and among its congeners. Similarly, this is the first known use of ITS-1 direct sequence variation to assess genetic structure in unionid bivalves at any taxonomic level. The results demonstrate that DNA sequence polymorphism in the COI and ITS-1 regions were effective in the characterization of intraspecific phylogeographic structure among *L. subviridis* populations and in identifying phylogenetic relationships among selected *Lasmigona* species.

Information regarding *L. subviridis* population structure suggests that this species should be viewed as a series of genetically differing populations with definable

phylogeographic structure. Bivalves inhabiting the Susquehanna and Potomac Rivers (localities 1–4) were differentiated from those in the southern portion of the species' range as indicated by diagnostic sequence differences in the ITS-1 region (Fig. 3). In addition, sequence variation in the COI region suggested a lack of gene flow between *L. subviridis* populations in different tributaries of the same river (localities 1–2 and 6–7) and adjacent river systems (locality 5) (Fig. 1). The lack of absolute congruence between the nuclear and mtDNA sequence variation within *L. subviridis* may reflect the differing evolutionary dynamics for the two regions (mitochondrial coding vs. nuclear noncoding) and that the reproductive isolation documented is due to relatively recent events.

Two forms of evidence suggest that the genetic divergence observed among *L. subviridis* populations and the inferred reproductive isolation are of recent origin. First, the divergence among populations is limited, with less than 1% sequence divergence observed in both DNA regions; a relatively modest level when compared to the degree of interspecific divergence (Table 3). Second, the absence of divergence between bivalves from the interior basin drainage (locality 9) and the Atlantic slope populations suggests that the event that introduced *L. subviridis* to the Atlantic slope was also recent compared to the time of divergence among the other *Lasmigona* species.

The influence of environmental change on the distribution of organisms and on the barriers between populations and species has long been recognized as significant in geographical allopatric speciation (Mayr 1963). Therefore, historical rather than contemporary dispersal may best elucidate current phylogeographic patterns of genetic diversity (Bernatchez & Wilson 1998). From the global magnitude of disturbances caused by Pleistocene glaciation, it is reasonable to assume that advance and retreat of continental ice had significant impacts on dispersal and ultimately the genetic composition of freshwater species even in areas outside the immediate glacial influence (Bernatchez & Wilson 1998). Glacially induced climate changes, including the associated sea-level and oceanographic changes, represent abiotic, extrinsic factors that could have influenced faunal distributions (Hallam 1983; Cronin & Schneider 1990) along the mid-Atlantic coast of North America. Thus, we hypothesize that a large-scale vicariant process, in the form of sea-level rise, served as an isolating mechanism between the northern and southern populations of *L. subviridis* surveyed in this study.

The Appalachian Mountain system of eastern North America forms a sharp faunistic division between organisms inhabiting drainages of the interior basin and that of the Atlantic slope (Ortmann 1913). Unionid bivalves constitute one group of organisms whose distributions illustrate this division in fauna. However, some unionid species have crossed the divide from the west into Atlantic slope drainages and spread north and south from the point of entry. Ortmann (1913) proposed that *L. subviridis* developed in the western mountain streams flowing to the continental interior from *L. compressa* and subsequently crossed the divide into the Atlantic slope region by headwater capture. Although no differentiation was observed between the interior (Site 9) and the southern Atlantic slope drainages (Sites 5–8) of *L. subviridis*, this evolutionary scenario is consistent with the phylogenetic analyses presented in this study between *L. subviridis* and *L. compressa* (Figs 2 and 4; Table 2).

Seas were probably closest to their lowest level during the southernmost expansion of Wisconsinan glaciation. During this time (> 15 000 years ago) the coastline of eastern North America existed well to the east, nearly to the Continental Shelf (Hocutt *et al.* 1986). All Chesapeake Bay streams south to the James River (localities 1–7) were tributaries to the Greater Susquehanna River, which enlarged and waned with vacillating sea level (Hocutt *et al.* 1986). Coastal flooding from heavy rainfall probably provided periodic means for interdrainage dispersal by host fishes (Hocutt *et al.* 1986) and thus allowed gene flow among the Atlantic slope rivers. With the retreat of Wisconsinan glaciation, occurring between 15 000 and 8000 years ago (Dawson 1992), and the subsequent saltwater intrusion, *L. subviridis* populations inhabiting the headwaters of the rivers to the south of the Potomac and Susquehanna Rivers (localities 5–8) could have become reproductively isolated. Physiological constraints on the fish host imposed by saline or brackish water could limit dispersal (and thus gene flow) between drainages (Ortmann 1913; Johnson 1970; Kat & Davis 1984) particularly in headwater species such as *L. subviridis*. It seems improbable that a host fish would be capable of making migrations from the headwaters of Atlantic slope streams through brackish to saline waters and ascend to the headwaters of an adjacent river system all during the period of glochidial attachment to the host. If this reproductive isolation scenario were accurate, the latest sea level rise is the only event that could have isolated the Rappahannock River (and rivers south) from the Potomac and Susquehanna Rivers. Therefore, the reproductive isolation between the northern and southern populations of *L. subviridis* would have occurred less than 15 000 years before the present time. This time frame is insufficient for large-scale sequence divergence to have occurred.

In light of the host fish migrations that must have taken place to permit the expansion of *L. subviridis* along the Altantic slope region, the presence of diagnostic mtDNA haplotypes between tributaries within the Susquehanna (localities 1 and 2) and James Rivers (localities 6 and 7) presents a conundrum. The absence of gene exchange between these pairs of localities suggests either the absence of a traditional dispersal mechanism or, alternatively,

demographic influences. We propose two possible explanations for this finding, based on host fish differentiation and the potential lack of a host fish. First, it is possible that the discrete *L. subviridis* populations use distinctly different host fish. The host fish could also exhibit limited vagility such that migration between tributaries is absent or its migrations could be temporally discordant with unionid reproduction. A second explanation is that under certain environmental and/or demographic circumstances, glochidia transform within the bivalve without the direct aid of a host. Recently, three independent sources researching the life history of *L. subviridis* have observed that the bivalve may be able to forego the parasitic stage (G. T. Waters, US Fish and Wildlife Service; W. Lellis, US Geological Survey; R. F. Villella, US Geological Survey, personal communications). Perhaps *L. subviridis* populations experiencing low population density, low water levels, or the absence of the host have the ability to transform their own glochidia into juvenile bivalves.

An alternative, and completely plausible, hypothesis for the observed distribution of COI haplotypes is lineage sorting (Avise *et al.* 1984). Perhaps population sizes reached critically low levels effectively bottlenecking the populations. In the absence of gene flow, complete lineage sorting could have occurred in some headwater streams. Genetic markers (both mitochondrial and nuclear in origin) which identify and describe population level genetic variation are needed for *L. subviridis* and other unionid species to address this and other demographic-based issues.

Phylogenetic utility of ITS-1 and COI in Lasmigona

Contemporary molecular systematics studies have demonstrated that certain genes (or DNA regions) are more suitable than others for reconstructing evolutionary relationships among taxa at particular levels of divergence (Simon *et al.* 1994). Noncoding DNA sequences are often more variable than coding regions and their use could be considered more appropriate at lower taxonomic levels (Smith & Klein 1994; Savolainen *et al.* 1997). Internal transcribed spacer regions of rDNA have been shown to evolve rapidly and be useful in inferring phylogenetic relationships at the generic and intrageneric levels in plants (Baldwin 1992), at the species level in the blennioid fish family (Stepien *et al.* 1993), and at interspecific and intraspecific levels in salmonids (Phillips & Oakley 1997). In the present comparison of unionid bivalve species in the genus *Lasmigona*, the ITS-1 region was less variable at the interspecific level (0.2–5.1% sequence divergence minus indels) than the COI coding region (3.8–14.9%). In contrast, the ITS-1 region was more variable than the COI coding region at the intraspecific level in *L. subviridis*, as seven positions (three transversions and four indels) were found to vary between the two major genotypes while a total of two substitutions (and no indels) were observed in the relatively conserved COI coding region. Notwithstanding the lack of knowledge concerning the evolutionary dynamics of the noncoding ITS region studied, the hierarchical structure contained in the DNA sequences appears suitable for phylogeny estimation. Indeed, the interspecific ITS-1 distances presented here were statistically congruent ($r = 0.84$) with distances obtained from the COI region of mtDNA. One caveat is that the presence of a large number of indels in the ITS-1 region between some distant taxa may render sequence alignment difficult.

Among-site rate heterogeneity exists within protein coding genes because of structural and functional constraints (Li & Graur 1991). For *L. subviridis*, intraspecific variation in COI was limited to two variable sites resulting in three mtDNA haplotypes. Interspecifically, however, greater genetic differentiation was observed among the four *Lasmigona* species as divergence estimates ranged from 3.8% to 15.0%. These findings correspond well to the levels of sequence divergence observed in the COI region by Roe & Lydeard (1998) in a phylogenetic comparison among unionids in the genus *Potamilus*. In that study, COI sequence variation between species ranged from 1.2% to 14.5% and phylogenetic analyses suggested that the genus was polyphyletic. In addition, geographical populations of the species *P. inflatus* exhibited genetic distinctiveness (2.6% sequence divergence) and were recommended for separate species status.

Among species in the genus *Lasmigona*, COI appears to be evolving at a faster rate than the nuclear rDNA region. This is noteworthy given that ITS-1 is a noncoding region and COI is a relatively conserved coding mtDNA gene in terms of its amino acid evolution (Simon *et al.* 1994). This latter observation may be due to the generally stronger selective pressures on the coding region. In conserved coding gene regions, transitions should predominate during the initial stages of divergence. In this comparison, the overall Ts/Tv ratio at the COI region was 5.0 suggesting a paucity of multiple substitutions. The high percentage of third position substitutions provides assurances that the region sequenced is not a nuclear pseudogene. However, the high percentage of second position substitutions observed relative to the number in the first position is unusual and defies a simple explanation. This trend was not observed in the genus *Potamilus* by Roe & Lydeard (1998). The low Ts/Tv ratio for ITS-1 (0.5) suggests the absence of a transition bias in the region and is consistent with the hypothesis that transversion substitutions are twice as likely to occur at a given location (Schlötterer *et al.* 1994) under selective neutrality. Alternatively, the apparent conservation in the relatively short

ITS-1 (≈ 600 bp) may result from indirect constraints associated with the two highly conserved rDNA genes flanking the region. Additional research comparing the evolutionary rates between the two regions in other genera is needed to better understand these findings.

Conservation implications

The genetic variation observed among populations of *L. subviridis* could be considered minor genetic differences because the observed differentiation may not correlate with phenotypic divergence, or represent obvious adaptive significance. However, the base substitutions and indels observed not only can be used as tags to provide insight into population dynamics, but they provide the advantage of being heritable and able to provide insight into evolutionary processes shaping the individual–population–species continuum. If this genetic differentiation is simply discounted as 'minor genetic differences among populations of invertebrates' that do not justify 'biological conservation at the population level' (Neves 1997), the potential for future evolutionary change within this species could be compromised. The specific concern is that the short-term goals (e.g. relocations to avoid extinction from zebra mussel infestation) could result in management programmes that undermine what we believe should be the long-term objective: maintaining evolutionary potential. If discrete populations (or cryptic species) are a low priority, then convenience, rather than biological reality, becomes the guiding principle of taxonomy, and there is little incentive to account for and describe the genetic diversity existing in freshwater bivalve populations.

If homogenization of populations becomes an acceptable management option, it has immediate implications for conservation due to the critical role taxonomy plays in the implementation of the US Endangered Species Act (ESA), particularly regarding invertebrates. Freshwater bivalves and other invertebrates are generally not recognized at the population level as units eligible for protection and management under the ESA. Invertebrate populations that constitute an 'important component in the evolutionary legacy of the species' can only be protected under the ESA if the entire species is listed or if the observed differentiation warrants a subspecific designation. Similarly, if a segment of the species' range warrants removal from the ESA list, the entire species or subspecies must be removed from protection. In the absence of ESA legislation to protect intraspecific groups of freshwater bivalves, systematists are forced into a 'splitter' mentality to isolate and elevate (to subspecies) geographical populations that warrant protection. If a goal of conservation biology is to preserve genetic integrity and evolutionary potential, recognition and protection must be mandated for intraspecific differentiation within bivalves.

We endorse the widespread opinion that decisions to protect a species under the ESA should be based on investigations of life history, population dynamics, and systematics. Freshwater bivalve systematic relationships should be determined using shell morphology, soft tissue (ultrastructure) anatomy, and molecular data. However, molecular systematics is a tool with the demonstrated ability to identify evolutionarily divergent lineages in rare unionid bivalves that other methodologies overlooked (Hoeh *et al.* 1995; Lydeard *et al.* 1996; Mulvey *et al.* 1997; Roe & Lydeard 1998). In the present study, a zone of discontinuity was identified between the Rappahannock and Potomac Rivers that suggests that the populations in the northern and southern portions of the range appear to be heading down different evolutionary paths, which may lead to allopatric speciation. As *L. subviridis* inhabits headwater streams, it is unlikely that the host would migrate from the upper reaches of one major river to another as restrictions of the host by saline or brackish water will limit dispersal between drainages (Ortmann 1913; Johnson 1970; Kat & Davis 1984). Given the current levels of the Atlantic Ocean it is unlikely that gene exchange among northern and southern populations of *L. subviridis* could be re-established until the next major ice age. We therefore suggest that the northern and southern populations, which are reproductively isolated and constitute evolutionarily significant lineages, be treated as separate management units. Results from the COI region suggest that relocations should be avoided between tributaries of the same drainage. Although the differentiation was minimal (2 bp among the haplotypes) the diagnostic nature of the variation suggests that these populations may have been reproductively isolated for thousands of generations.

The research presented here suggests that species of unionid bivalves with populations distributed among discontinuous habitats (e.g. Atlantic slope drainages) should be considered potentially evolutionarily distinct unless proven otherwise. Judgements such as these will often require the use of contemporary molecular genetic methods to document gene flow patterns and the presence of evolutionarily significant lineages (e.g. Hoeh *et al.* 1995). Both regions of DNA surveyed in this study appear to provide sufficient phylogenetic (or coalescence) signal for use in delineating systematic relationships in freshwater bivalves.

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Tim King's research emphasis involves the application of molecular genetic markers to questions in population genetics, phylogeography, and phylogenetics of declining species with the purpose of identifying evolutionarily significant lineages. This contribution is yet another effort to assist natural resource managers with guidance in identifying appropriate units of conservation. Mike Eackles and Branimir Gjetvaj are a technician and postdoctoral fellow, respectively, working in King's laboratory. Walter R. Hoeh is Assistant Professor of Biological Sciences at Kent State University and his research interests cover molecular systematics and evolutionary biology of invertebrates, specializing in unionid bivalves.
