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APPLICATION OF MOLECULAR GENETIC MARKERS TO CONSERVATION OF FRESHWATER BIVALVES

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ABSTRACT Freshwater bivalves (Unionacea) are among the most endangered faunal elements in North America. Molecular genetic studies have much to offer conservation efforts directed to this declining fauna. Molecular genetic data can provide information needed to identify evolutionarily significant units, resolve taxonomic ambiguities, describe population structure, evaluate impacts of habitat fragmentation and reduced gene flow among populations, reconstruct phylogenetic relationships, clarify fish host-glochidia relationships, and provide evidence in legal actions. Molecular genetic techniques and their application to freshwater bivalves are reviewed.

KEY WORDS: Freshwater bivalves, Unionacea, genetics, conservation, molecular markers

INTRODUCTION

Freshwater bivalves are among the most endangered of animal groups. Reductions in the number of species and in the abundance of freshwater bivalve populations have been reported worldwide (Bogan 1993, Williams et al. 1993, Ziuganov et al. 1994, Lydeard and Mayden 1995, Williams and Neves 1995, Abramovitz 1996). Dam construction, channelization, pollution, commercial exploitation, and introduction of exotic species contribute to extinction, population fragmentation, and reduction in population sizes. Changes in dispersal and gene flow between populations and reduced effective population size occur concomitantly with these environmental perturbations and may lead to loss of genetic diversity. Therefore, in addition to concerns about the outright loss of freshwater bivalves, conservation strategies must consider the potential ecological and evolutionary impact of changes in genetic characteristics.

Genetic diversity has been shown to be relevant to population health and probability of persistence (O'Brien and Evermann 1988, Quattro and Vrijenhoek 1989). Numerous studies of marine bivalves report a positive relationship between genetic diversity and surrogate measures of fitness such as growth (Garton et al. 1984, Koehn et al. 1988), fecundity (Rodhouse et al. 1986), and survival (Diehl and Koehn 1985). Although this relationship has not been extensively studied in freshwater bivalves, a comparable relationship may be expected. Genetic diversity should be conserved because of its immediate contribution to fitness-related traits, and because genetic diversity is an essential component of adaptation and evolutionary success.

Molecular genetic markers provide powerful tools to investigate ecology, demography, biogeography, and evolutionary history. Genetic markers can be used to determine if a group of organisms constitutes a species, subspecies, or population. Determination of species status has direct and immediate application to conservation decisions as well as being of general interest to evolutionary biology. More specifically, conservation biologists interested in freshwater bivalves are concerned with several issues that can be approached from a molecular genetic perspective. These issues include identification of Evolutionarily Significant Units (ESUs) and Management Units (MUs), systematics and taxonomy, spatial patterns of variation (intraspecific phylogeny), gene flow, hybridization, inbreeding, bivalve-fish host relationships, and forensics. Currently, there are few molecular genetic studies of freshwater bivalves.

Several recent publications provide excellent summaries of molecular genetics and applications to conservation issues (Avise 1994, Moritz 1994a, O'Brien 1994, Avise and Hamrick 1996, Hillis et al. 1996, Ferraris and Palumbi 1996, Haig 1998). Here, we provide only a brief technical review and summarize information regarding molecular genetic markers and freshwater bivalves. Our focus is on conservation issues of freshwater bivalves and molecular approaches that may help address these issues.

MOLECULAR GENETIC MARKERS

Morphological and physiological phenotypes are often under complex polygenic control, subject to environmental perturbations, and may be direct targets for selection. In contrast, molecular genetic markers (protein and DNA) have simple genetic underpinnings and most can be considered to behave as neutral markers. Information contained in these molecules can be used to evaluate population and evolutionary processes. Selection of appropriate molecular genetic markers, from the many that are available, is critical. Of primary importance, the marker must provide genetic variation appropriate to the question. Secondary considerations include time required to process samples, specialized equipment or training, and cost (Table 1).

Protein Electrophoresis

Protein electrophoresis provides a convenient, reliable, and cost-effective tool to study population genetic processes. A general overview of electrophoretic procedures and specific buffers and staining methods can be found in Richardson et al. (1986) and Hillis et al. (1996). Proteins are separated on or in a supporting

TABLE 1.

Comparison of methods available for molecular genetic studies and their routine application and relative cost.

Methods	Application[a]	Genome	Number of Loci	No. Individuals	Cost
Protein electrophoresis	CP, CRS	Nuclear	Many	Many	$
Restriction fragment length Polymorphism (RFLP)	CP, CRS	mtDNA, Nuclear	Few	Many	$$
Ramdon amplified Polymorphic DNA (RAPD)	CP, CRS	Nuclear	Many	Many	$$
Microsatellites	CP	Nuclear	Few to many	Few to many	$$
DNA Sequencing	CP, CRS, DRS, DP	mt DNA	Few Nuclear	Few	$$$

CP—conspecific populations, CRS—closely-related species, DRS—distantly related species, DP—deep phylogenetic reconstruction.

medium (e.g., starch, polyacrylamide, and cellulose acetate) by charge differences associated with changes in amino acid sequence and/or by size. Specific enzymes or proteins are visualized using histochemical stains. The underlying genetic basis for most banding patterns is well established and interpretation of banding patterns follows rules of simple Mendelian inheritance. One or more loci may be visualized and each locus may have one or more alleles. Thus, protein electrophoresis can provide information for numerous independent loci throughout the genome for genetic study. The following information is routinely obtained with allozyme studies: number of alleles per locus, percent polymorphic loci, heterozygosity, tests for fit of data to random mating expectations, estimates of population differentiation (F-statistics, contingency tables), and genetic distance and identity measures. Numerous programs are available for analysis of electrophoretic data including BIOSYS-1 (Swofford and Selander 1981) and others described in Hillis et al. (1996).

DNA Approaches

The application of DNA-based molecular genetic markers involves a consideration of nuclear versus mitochondrial genomes. The mitochondrial genome of bivalves, like that of other animals, is about 18 kilobase (kb) pairs in length and composed of about 37 genes. These genes code for tRNAs, rRNAs, and proteins involved in electron transport and oxidative phosphorylation. The mitochondrial genome represents a single, complex linkage group. Although mtDNA is inherited through maternal lines in many animals, in bivalves gender-specific genomes have been reported and the pattern of inheritance is described as doubly uniparental inheritance (DUI) (Skibinski et al. 1994, Zouros et al. 1992, 1994). Male and female mtDNA genomes are nonrecombining and highly divergent (Hoeh et al. 1996, Liu et al. 1996b). Regions of the mtDNA differ in their rates of divergence and in their utility for studies at different hierarchical levels of analysis from populations to systematics.

The nuclear genome is not as well studied as the mitochondrial genome. Single-copy gene sequences can be obtained via the polymerase chain reaction (PCR) using specially designed primer pairs (e.g., Karl and Avise 1993). Nuclear rRNA genes consist of tandemly repeated units (Fig. 1a) and have been examined in freshwater bivalves (Liu and Mulvey, unpubl). The repeated units may occur in high copy numbers and may be dispersed throughout the genome. Despite the large copy number there is a high degree of homogeneity in these regions (Hamby and Zimmer 1992). In a study of freshwater bivalves in the genus *Elliptio,* Liu and Mulvey (unpubl) found that the internal transcribed spacer rejoins (ITS1 and ITS2) were highly variable whereas the rRNA coding regions (18S, 5.8S, and 28S) were conserved. Segments of the rRNA repeat unit may be useful in studies of freshwater bivalves including populations studies (spacers) and phylogenetic reconstruction (rRNAs).

DNA Extraction

Foot, mantle, gill, gonad, and muscle tissue have been used to obtain DNA for analysis. Fresh, frozen, and ethanol-preserved specimens are good sources for DNA samples. Nondestructive sampling methods have been described for bivalves (Stiven and Alderman 1992, Berg et al. 1995). These methods will be especially useful when endangered or threatened species are studied. DNA extraction from freshwater bivalve tissues can be accomplished using a variety of methods including standard phenol-chloroform (Sambrook et al. 1989), and chelex (Walsh et al. 1991). Methods have recently been described for use of formalin-fixed specimens for DNA isolation and PCR amplification (Shedlock et al. 1997). Thus, historical museum collections of freshwater mussels may now be accessible to modern molecular genetic analysis.

Polymerase Chain Reaction

The PCR takes advantage of thermo-stable DNA polymerases to amplify DNA sequences from template DNA to provide large quantities of specific sequences. PCR requires only a small amount of template DNA; thus, this approach is well suited to endangered species and conservation. PCR products can then be used in subsequent analyses as described below. PCR requires primers, which are short DNA fragments, to initiate DNA synthesis. Primers may be random or gene-specific. Primer development was a limitation in the application of this technique early in its development but primer design has been greatly facilitated by the availability of sequence data (Genbank, EMBL, and other resources).

Numerous primers have been successfully applied in studies of freshwater bivalves (Table 2). As illustrated in Figures 1a, b, and c, primer pairs can be selected to obtain PCR products for several regions, to select the size of the fragment, and to select for regions that are "fast-evolving" or "slow-evolving." Additionally, many of the "universal" or kit primers may be useful in studies of freshwater bivalves but have not yet been widely applied.

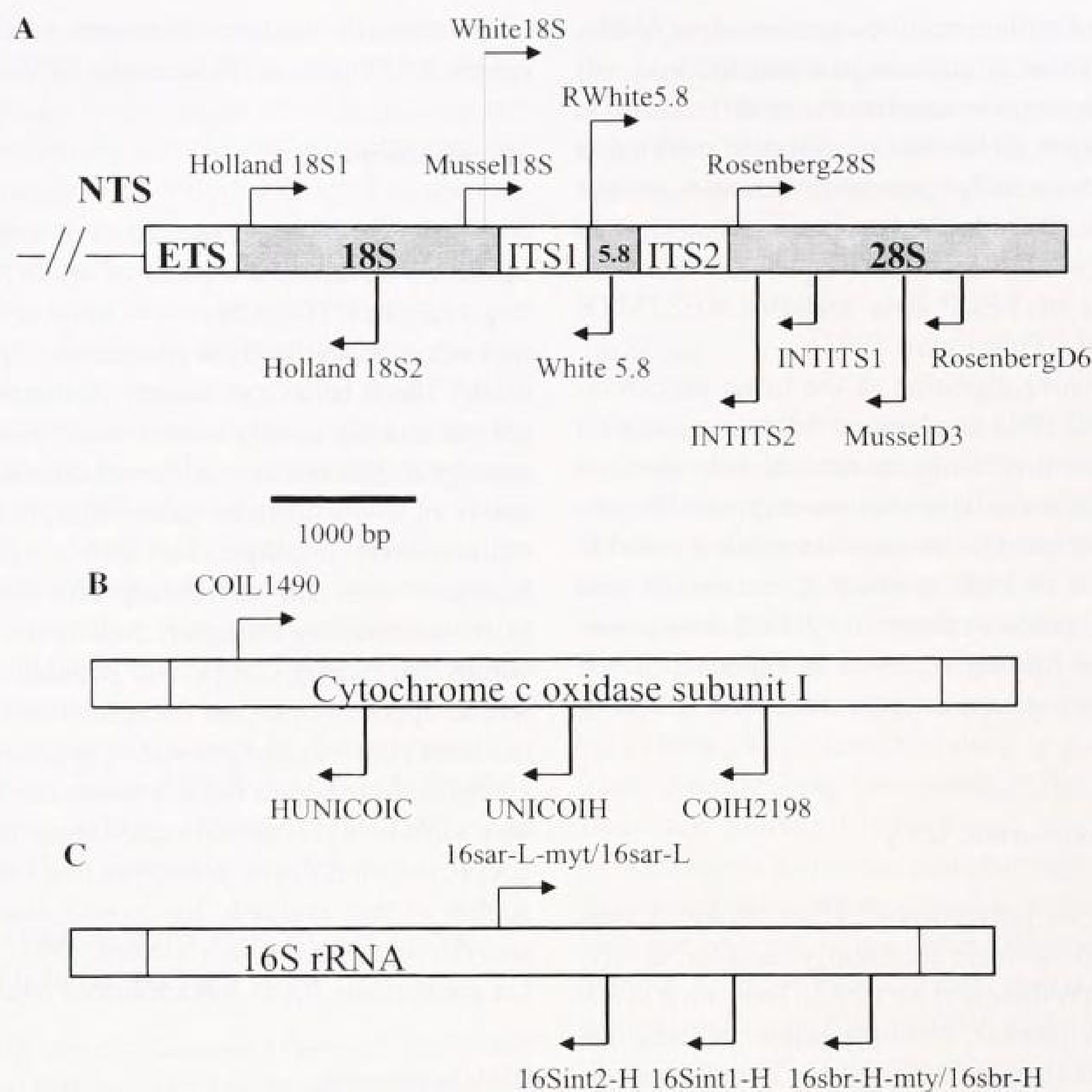

Figure 1. Location of primers used for PCR amplification of DNA for freshwater bivalves. (a) rRNA array, (b) COI, (c) 16S rRNA. Primers are listed in Table 2.

DNA Markers

Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphisms (RFLP) are obtained when restriction enzymes cleave double-stranded DNA. These enzymes have specific recognition and cleavage sites usually of four, five, or six base pairs. Data are generated by digestion of DNA with a series of restriction enzymes and size-based separation of the resulting fragments using gel electrophoresis (agarose or acrylamide). The number and size of the resulting fragments

TABLE 2.

Primers that have been used in DNA sequencing studies of freshwater bivalves.

Gene Segment	Primer Label	Primer Sequence	Reference
COI	UNICOIH	5′-TCA GCA ACC AAC CCA GGA G-3′	Roe & Lydeard (unpubl)
	HUNICOIC	5′-AAC AAC ACT CTC TAC CAA AG-3′	Roe & Lydeard (unpubl)
	COIL 1490	5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′	Folmer et al. 1994
	COIH 2198	5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′	Folmer et al. 1994
16S rRNA	16sar-L	5′-CGC CTG TTT ATC AAA AAC AT-3′	Palumbi et al. 1991
	16sbr-H	5′-CCG GTC TGA ACT CAG ATC ACG T-3′	Palumbi et al. 1991
	16sar-L-myt	5′-CGA CTG TTT AAC AAA AAC AT-3′	Lydeard et al. 1996
	16sbr-H-myt	5′-CCG TTC TGA ACT CAG CTC ATG T-3′	Lydeard et al. 1996
	16Sint1-H	5′-GAAA ARG TAA AGY TCC GC-3′	Lydeard et al. 1996
	16Sint2-H	5′-RGR TTG CCC CAA TCH HHC-3′	Lydeard et al. 1996
18S rRNA	Holland 18S1	5′-GCC AGT AGC ATA TGC TTG TCT C-3′	Adamkewicz et al. 1997
	Holland 18S2	5′AGA CTT GCC TCC AAT GGA TCC-3′	Adamkewicz et al. 1997
ITS1	Mussel18S	5′TCC CTG CCC TTT GTA CAC ACC G-3′	Liu & Mulvey (unpubl)
	WHITE18S	5′-TAA CAA GGT TTC CGT AGG TG-3′	White et al. 1994
	WHITE5.8	5′AGC TRG CTG CGT TCT TCA TCG A-3′	White et al. 1994
ITS2	RWHITE5.8	5′-TCG ATG AAG AAC GCA GCY AGC T-3′	White et al. 1994
	INTITS2	5′-TTT TCC CTC TTC ACT CGC CGT TAC-3′	Liu & Mulvey (unpubl)
28S rRNA	Rosenberg28S	5′-GCG GAG GAA AAG AAA-3′	Rosenberg et al. 1994
	INTITS1	5′-CGT GGC AAT CAA CCC GAG GAA AGT-3′	Liu & Mulvey (unpubl)
	MusselD3	5′-CCT TCT CAG GCA TAG TTC ACC ATC-3′	Liu & Mulvey (unpubl)
	RosenbergD6	5′-CTA CTA CCA CCA AGA TCT GC-3′	Rosenberg et al. 1994

depend on the number and distribution of recognition sites. Variation in fragment patterns arises from base pair substitutions, insertions or deletions, sequence rearrangements, or differences in the overall size of the target DNA. Data consist of restriction fragment lengths, which are scored as present or absent or restriction sites which are map locations. In the latter case, scores consist of the presence or absence of recognition sequences. Software available for the analysis of RFLP data includes RESTSITE (Miller 1991) and RESTML (Felsenstein 1993).

Many RFLP studies involve digestion of the intact mitochondrial genome. Mitochondrial DNA is obtained following isolation and purification of mitochondria using cesium-chloride gradient centrifugation prior to digestion with restriction enzymes. The development of PCR and associated techniques has made it possible to apply the RFLP method to PCR products from nuclear and mitochondrial genomes. A general scheme for RFLP data generated for the ITS1 region of *Elliptio* is shown in Figure 2. RFLP data are used to evaluate population differentiation and to reconstruct phylogenies.

Random Amplification of Polymorphic DNA

Random amplification of polymorphic DNA (RAPD) uses short (approximately 10 bp) primers to amplify random, anonymous sequences with PCR (Williams et al. 1990). Thus, no *a priori* knowledge of sequences is needed. A single primer is used and PCR products are fragments flanked by sequences complementary to the primer. Data consist of presence or absence scores for size-separated fragments on polyacrylamide or agarose gels. Polymorphisms display dominant-recessive patterns. Numerous primers are commercially available to facilitate screening for informative markers. This method is particularly useful, when crosses are done to verify inheritance patterns. RAPD markers are usually applied to intraspecific analysis. Software available for the analysis includes RAPDistance (Felsenstein 1993).

Microsatellite DNA

Microsatellite loci are a class of highly polymorphic markers identified by tandem repeats of short (2–4 bp) DNA sequences (e.g., AC_n or CTG_n, where n = number of tandem repeats). Variation arises primarily from changes in copy number of the repeated motif. These behave as simple co-dominant Mendelian polymorphisms and are readily scored when microsatellite fragments generated via PCR are size-separated on nondenaturing 6% polyacrylamide or 3–5% agarose gels. Multiple loci may be analyzed simultaneously (multiplexed) with a single PCR reaction when fragment sizes are sufficiently different to allow identification. Microsatellites are especially well suited to study genetic variation within and among conspecific populations. A limitation to widespread application of the microsatellite technique is the difficulty in identifying loci and generating appropriate primer pairs for PCR amplification. Additionally, primers are usually species-specific so they must be developed for each application. Microsatellite analyses provide multilocus genotypes that can be analyzed in a manner similar to that available for protein electrophoresis. Software for analysis includes Misat (Nielsen 1997), and MicroSat (available via anonymous ftp at lotka.stanford.edu/microsat.html).

DNA Sequencing

DNA sequence data provide the most direct assessment of genetic characteristics. Methods discussed above are indirect (protein electrophoresis) or incomplete (RFLP) approaches to genomic sequences. DNA sequence data are becoming widely used because of the availability of PCR techniques and automated DNA se-

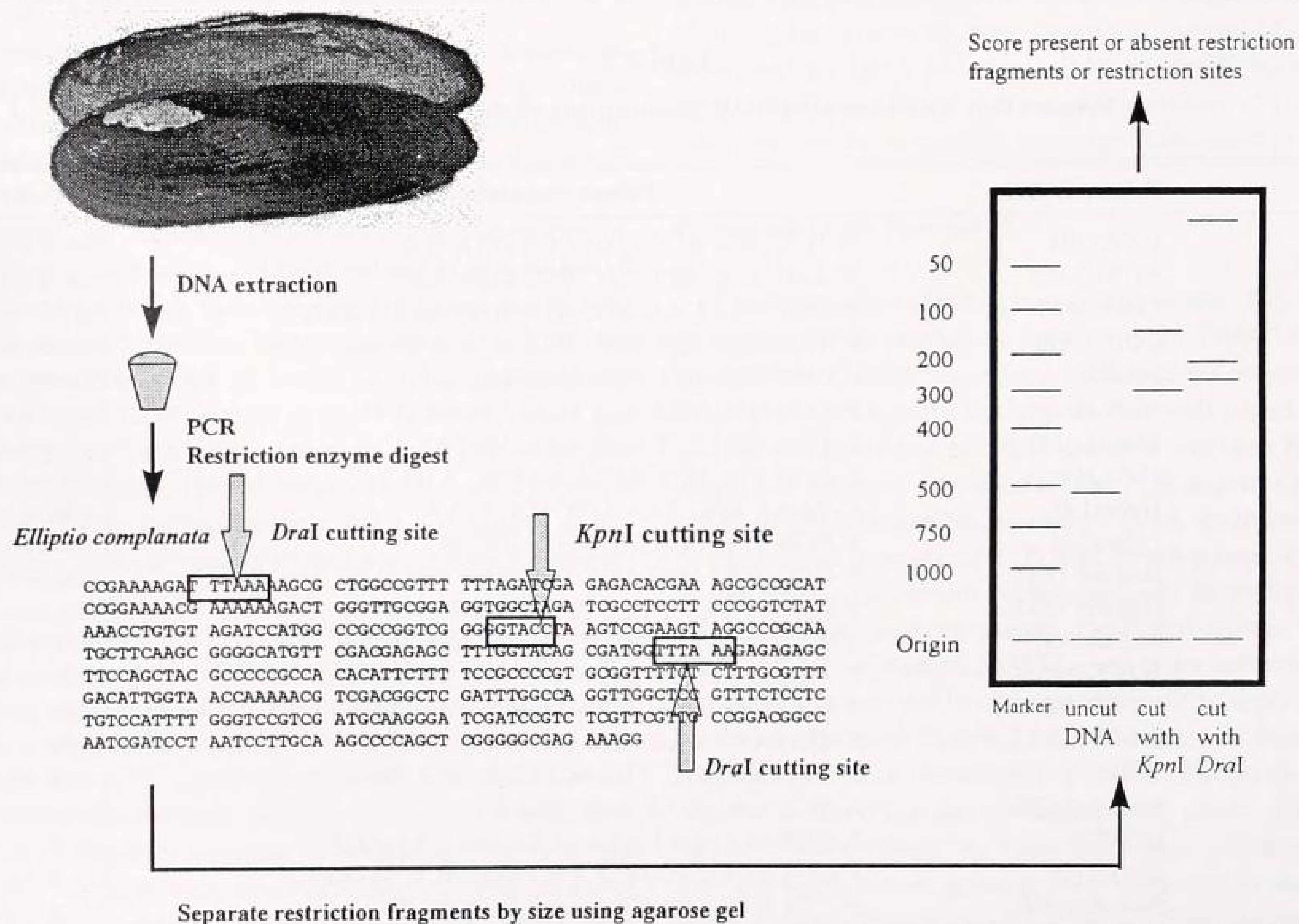

Figure 2. Overview of RFLP analysis of the ITS1 region of *Elliptio* cut with restriction enzymes *Kpn*I and *Dra*I.

quencing methods. Unlike the short primers used for RAPD analysis, primers used to generate sequence data are usually 20–30 bp. This length ensures specificity to the target DNA segment in the PCR. Sequencing reactions usually involve chain termination with ddNTPs to generate fragments with different lengths at termination. Fragments are separated in polyacrylamide gels and read from autoradiograms or with automated scanning devices.

One potential difficulty with DNA sequence analysis is sequence alignment. Sequence data from different samples must be homologous to allow appropriate comparisons to be made. Alignment is usually not too problematic for protein-coding genes but becomes increasingly difficult for rRNA (especially loop segments) and noncoding regions. Sequence alignment can be facilitated with programs such as Sequencher™ and ClustalV as described in Hillis et al. (1996). Loop or noncoding regions can be aligned with the help of secondary structure programs such as RNAviz (De Rijk and De Wachter 1997) and DCSE (De Rijk and De Wachter 1993). Alignments should be verified by visual inspection. Following successful alignment of homologous sequences, data can be analyzed using PAUP*4.0 (Swofford 1998), PHYLIP (Felsenstein 1993), MEGA (Kumar et al. 1993), and Hennig86 (contact biodl@wuvn.gwu.edu).

CONSERVATION APPLICATIONS FOR MOLECULAR GENETIC DATA

Application of molecular genetics to conservation of freshwater bivalves has great potential that has only just begun to be realized. Below we review available literature regarding the application of molecular genetics to freshwater bivalves and speculate on some uses for the future.

Evolutionarily Significant Unit

The concept of the evolutionarily significant unit (ESU) was advanced by Waples (1991) and later Moritz (1994b) to address the issue of genetic distinctiveness as recognized by the Endangered Species Act (ESA). The ESU concept integrates genetic, phenotypic, life history, ecological, and geographic information to identify units that are independent over evolutionary time. With the recognition of ESUs, conservation efforts are directed to the evolutionary legacy of the species. ESUs reflect long periods of genetic isolation and deep phylogenetic subdivisions. Management units (MU) represent more recent separations and shallower genetic subdivisions. Bowen 1998, and Roe and Lydeard (1998) provide extensive comment on the ESU concept and its application to molluscan conservation.

The ESU concept has rarely been explicitly applied to bivalves; however, as the following examples illustrate, this concept may have broad utility for conservation of freshwater bivalves. Roe and Lydeard (unpubl) argue for the recognition of ESUs in *Potamilus alatus* based on genetic data. Because genetic differentiation (based on DNA sequences) between the two extant populations from the Amite River and Black Warrior River was significant, Roe and Lydeard argue that they represent separate targets for conservation measures. Additionally, they suggested that specific designation be given to the Amite River form. Similarly, Liu et al. (1996a) recommended that *Pyganodon grandis* from the Arkansas River and South Platte River drainages be managed as separate units (MUs). Populations in these drainages exhibited significant genetic differentiation, especially for the male type mitochondrial genome. King et al. (1997) used an RFLP approach with the ITS1 and COI genes to assess genetic differentiation in populations of the green floater, *Lasmigona subvirdis*. They found significant differences between a Pennsylvania population and populations from West Virginia and North Carolina. King et al. (1997) argue for a conservative approach, based on an assumption of genetic distinction, in the management and conservation of freshwater bivalves.

Taxonomy

Recognition of an entity as an ESU or as a distinct species is controversial, and no simple criteria for the distinction are available. The complexity and duration of the life history of freshwater bivalves preclude the use of reproductive compatibility as a criterion for species delineation; therefore, there has been strong reliance on conchological characters. However, phenotypic plasticity and convergence of form have led to confusion in many taxonomic groups. Early malacologists clearly overestimated the number of taxa (Boss 1971); however, there is growing evidence that subsequent synonymizing has oversimplified the situation (e.g., *Elliptio*, Davis and Mulvey 1993).

Taxonomy forms the basis for legal protection under the ESA. Taxonomic uncertainty presents a problem for the conservation biologist because, although taxa may be endangered or threatened, without valid taxonomic names they cannot be evaluated for protection under the ESA. Additionally, phylogenetic distinction is often considered in species recovery plans and resource allocation. As Daugherty et al. (1990) stated, "good taxonomies are not irrelevant abstractions, but the essential foundations of conservation practice."

Molecular genetic approaches are often useful to clarify relationships. Ambiguous taxonomic placements often occur, where morphological criteria used to distinguish taxa exhibit phenotypic plasticity. For such approaches to be useful, it is necessary to estimate genetic differentiation within and between species. However, many endangered species are already too rare to permit sufficient sampling for such studies, and attempts to reconstruct historical demography are often complicated by the absence of independent estimates of variation prior to exploitation or bottlenecks. Additionally, it is difficult to draw conclusions when populations are allopatric. Comparative studies provide evidence for generalities regarding population genetic patterns and processes. A comparison of genetic diversity between common and rare species can provide useful guidelines for managers. Although genetic distance values alone cannot be used to establish taxonomic distinctions, these values can be compared with others to evaluate ranges of differentiation for populations, species, genera, etc. (see Table 3 for examples of allozyme data). Hoeh and Gordon (1996) provide cautionary comments on applications and implications of molecular data for taxonomic issues. Davis (1983) examined bivalves in the genera *Uniomerus* and *Elliptio* using allozyme electrophoresis and morphological characteristics. These genera are very similar conchologically. In addition to clearly distinguishing the two genera, the allozyme data revealed three cryptic species among individuals previously recognized as a single species, *U. tetralasmus* (Davis 1983). In this case, convergent morphologies obscured underlying genetic distinctions.

In contrast, a recent study of molecular genetic characteristics in the genus *Pleurobema* suggests that forms named on the basis of conchological features may not exhibit significant genetic differentiation (Kandl et al., unpubl). *Pleurobema reclusum* is described

TABLE 3.

Summary of genetic characteristics of freshwater bivalves determined from allozymes.

Taxon	No. Pop. or Species	No. Loci	P	H	Genetic Distance	Reference
Populations within species						
Anodonta cataracta	5				0.034 ± 0.038	Davis 1994
Elliptio complanata	11	8	0.357–0.500	0.041–0.084	0.065 ± 0.039	Davis et al. 1981 (cited in Davis 1994)
Lampsilis cariosa	3	11	0.636–0.818	0.260–0.318	0.071 ± 0.027	Stiven & Alderman 1992
Lampsilis radiata	5	7	0.071–0.357	0.004–0.041	0.018 ± 0.010	Kat & Davis 1984
Leptodea ochracea	2	11	0.273–0.364	0.051–0.100	0.018	Stiven & Alderman 1992
Pleurobema pyriforme	9	13	0.000–0.231	0.000–0.154	0.031 ± 0.029	Kandl et al. 1997
Species within genus						
Amblema	3	14	0.917		0.219 ± 0.025	Mulvey et al. 1997
Anodonta	3	14	0.113–0.357	0.028–0.107	0.457 ± 0.073	Kat 1983a (cited in Davis 1984, 1994)
Elliptio	7	14	0.280–0.470	0.094–0.146	0.210 ± 0.017	Davis 1981 (cited in Davis 1984, 1994)
Lampsilis	6	14	0.262–0.600	0.038–0.113	0.609 ± 0.478	Kat 1983b (cited in Davis 1984, 1994)
Megalonaias	2	14	0.617		All < 0.100	Mulvey 1997
Pleurobema	2	13	0.000–0.308	0.000–0.154	0.185 ± 0.045	Kandl et al.
Uniomerus	3	14	0.290	0.107	0.308 ± 0.165	Davis 1981 (cited in Davis 1984, 1994)

from the Ochlockonee and Suwannee rivers of Florida, *P. pyriforme* from the Apalachicola River system to the Suwannee River system of Florida and Georgia, and *P. bulbosum* from the Chipola River of Florida. Clench and Turner (1956) suggested that these taxa, *P. pyriforme, P. reclusum,* and *P. bulbosum,* represented a single polytypic form, *P. pyriforme*. The distinctiveness of *P. reclusum, P. bulbosum,* and *P. pyriforme* has been the subject of several works (Johnson 1970, Burch 1975, Heard 1979). Kandl et al. (unpubl) used allozyme, RFLP, and DNA sequence data (COI) to determine whether specimens from the eastern Gulf drainages exhibited genetic differentiation consistent with taxonomic designations or with the hypothesis of a single polytypic species. Little or no genetic differentiation was observed among forms recognized as *P. reclusum, P. bulbosum,* and *P. pyriforme* although significant genetic differentiation was found between these and *P. strodeanum* from the Escambia and Yellow rivers of Florida and Alabama. Kandl et al. suggest that *P. pyriforme* is a widely distributed, conchologically variable species and that includes *P. reclusum* and *P. bulbosum*. In 1998, "endangered" status was proposed for *P. pyriforme* (U.S. Fish and Wildlife Service 1998). The taxonomic revision suggested by the work of Kandl et al. would not alter the conservation status of *P. pyriforme*.

Population Structure—Intraspecific Phylogeography

Freshwater environments are spatially and temporally heterogeneous and genetic differentiation may reflect differences associated with local environments. River systems can be considered habitat "islands" on the larger continental landscape; each river occupies a distinct basin and is separated from other rivers by habitat unsuitable for bivalves. Riverine systems exhibit varying degrees of contemporary and historical connectedness and therefore opportunities for gene flow. Northern glaciated rivers may have been colonized as recently as 10,000 years ago. Southern rivers were unglaciated and generally support more total species and more endemic species than northern rivers. In addition to natural barriers, man-made impediments (e.g., dams and channelization) to gene flow occur on most major river systems in the U.S.

Habitat fragmentation and thus fragmentation of gene pools is common for freshwater species. The distribution of genetic variability among fragmented populations depends on the distribution of genetic variability before fragmentation, the size and number of fragments, the duration of fragment isolation, mating systems, and dispersal capabilities. Population fragmentation is likely associated with loss or redistribution of genetic variability and is a concern for conservation biologists attempting to manage the genetic legacy of freshwater bivalve species.

Molecular genetic methods provide an assessment of the amount and partitioning of genetic diversity necessary to evaluate the impacts of the current extinction crisis in freshwater mollusks. For many bivalve taxa, large-scale genetic assessments are no longer possible because there are practical and legal limitations on direct assessment of genetic patterns in rare or endangered species. Determination of genetic characteristics in related taxa may provide surrogate data to evaluate endangered populations and species. Freshwater bivalves display a wide range of genetic characteristics when evaluated for allozyme diversity (Table 3). Measures of genetic diversity were low for *Lampsilis radiata* (p = 7–36%, H = 0.004–0.041) and high for *L. cariosa* (p = 64–82%, H = 0.260–0.318). Other freshwater bivalves showed intermediate levels of genetic diversity.

For the conservation biologist, genetically differentiated, geographically isolated populations are especially problematic. It is difficult to know whether they represent variation within a species or distinct species. This situation is widespread among North American unionids and is accelerating as habitat becomes increasingly fragmented. Avise et al. (1987) provide a powerful phylogeographic approach in which geography is overlaid on an evolutionary tree to provide a landscape perspective of relationships. Data available for *Quadrula, Pyganodon,* and *Amblema* populations illustrate this approach for freshwater bivalves.

Little genetic differentiation was detected for four sequential beds of *Quadrula quadrula* along a 31-km stretch of the Ohio River. Berg et al. (1997) attributed this homogeneity to movement of bivalves with host fishes. For more geographically isolated populations (range from 80 km to >1,500 km downstream) they observed variation in allozyme frequencies consistent with a model of isolation-by-distance and possibly selection in response to environmental conditions. Genetic characteristics of the widely

distributed and conchologically variable *Pyganodon grandis* were described by Liu et al. (1996a). Significant genetic differentiation was noted between populations of *P. grandis* from the South Platte River and Arkansas River drainages. Mulvey et al. (1997) used allozyme and DNA sequence data and a phylogeographic approach with *Amblema* species. Figure 3 shows the distribution of COI haplotypes. The data confirmed the genetic distinction between *Amblema neislerii* and *A. plicata* and identified some difference in haplotype occurrence between eastern and western populations of *A. plicata*. The authors recommended that conservation efforts be directed to *A. neislerii* which has a range restricted to the Apalachicola River drainage in Georgia and Florida and narrow habitat requirements. This study also identified a genetically distinct but conchologically cryptic form, *A. elliottii*, from the Coosa and Conasauga Rivers. Additional work will be required to determine the conservation status of *A. elliottii*.

Phylogenetic Reconstruction

Evolutionary history can be estimated through the study of contemporary taxa. However, as suggested by the studies above, contemporary taxonomy and phylogenetic analysis can be at odds because phenotypically defined categories are not necessarily monophyletic. Adamkewicz et al. (1997) present a phylogenetic reconstruction for bivalve mollusks, including the freshwater bivalves, *Elliptio complanata* and *Utterbackia imbecillis*, based on DNA sequences for the 18S rRNA gene. Rosenberg et al. (1994) used about 150 bp of the D6 region of the large RNA subunit (28S) to construct relationships among bivalve and gastropod mollusks. This study included 20 freshwater bivalves. The data supported the distinction between Margaritiferinae and Ambleminae as discussed by Davis and Fuller (1981) but did not recognize a distinct Anodontinae clade advocated by these authors. Lydeard et al. (1996) used DNA sequence data for the 16S rRNA gene to construct a phylogenetic hypothesis for North American Unionaceans based on 29 taxa (Fig. 4). These authors argued for the recognition of families Margaritiferidae and Unionidae. The subfamily Anodontinae was clearly distinct from the remaining Unionidae; however, these data did not fully resolve relationships for the currently recognized Ambleminae and Lampsilinae. Additionally, these authors presented a previously unrecognized clade containing *Megalonaias* and *Quadrula*. Additional analyses of North American and global unionaceans are needed to clarify relationships and to serve as a basis for setting conservation priorities.

Hoch (1990) used allozyme and morphological data to generate phylogenetic hypothesis for 13 presumptive species of eastern North American *Anodonta*. Substantial revision was recommended with the three genetically differentiated clades given generic rank (*Anodonta, Pyganodon,* and *Utterbackia*). The molecular data suggested that ecophenotypic plasticity led to serious problems for traditional approaches to species determinations and evaluation of phylogenetic relationships. Umbo height relative to the hinge line, considered an important diagnostic characteristic, was shown to be misleading and did not identify monophyletic groups.

A cladogram derived from molecular genetic or other data can be used as a conservation tool. The terminal branches of the cladogram are extant taxa of potentially immediate conservation concern. Deeper branches indicate which groups are species-rich and which are species-poor. Although a controversial notion among conservation biologists, one strategy might be to focus conservation efforts on the smaller groups and assume that loss of one or two species from the species-rich clades would be less significant.

Bivalve-Fish Host Relationships

Conservation of freshwater bivalves is linked to fish conservation because of the complex life history and obligatory relationship of the glochidial stage. Regrettably, bivalve-fish host relationships are known for only about 25% of the North American bivalves and many proposed relationships are questionable (Hoggarth 1988, 1992). Failure to understand this critical life history relationship severely constrains conservation efforts. For example, the prob-

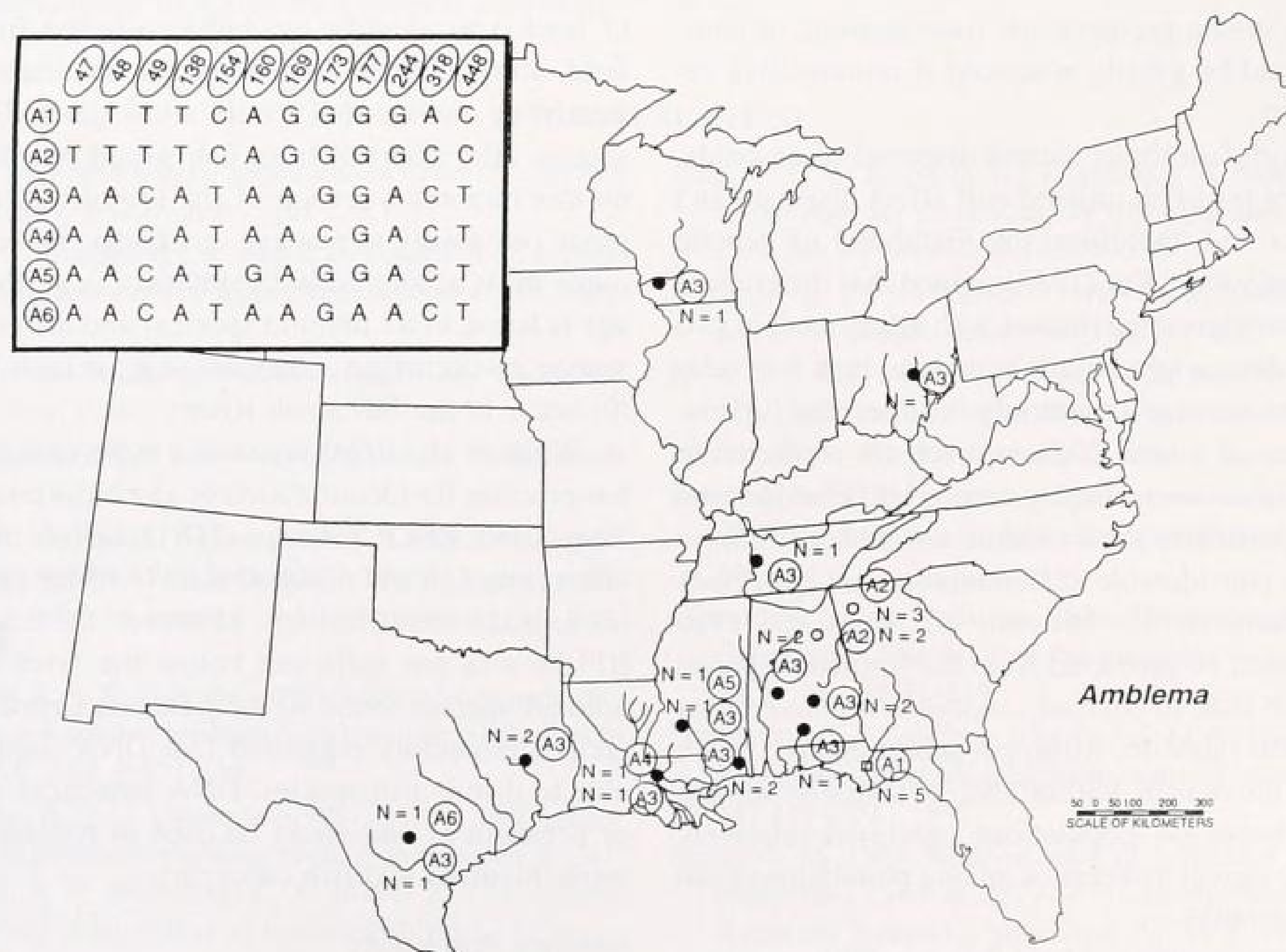

Figure 3. Distribution of haplotypes for the 16S rRNA gene for *Amblema plicata. A. neislerii,* and *A. elliottii.* (from Mulvey et al. 1997).

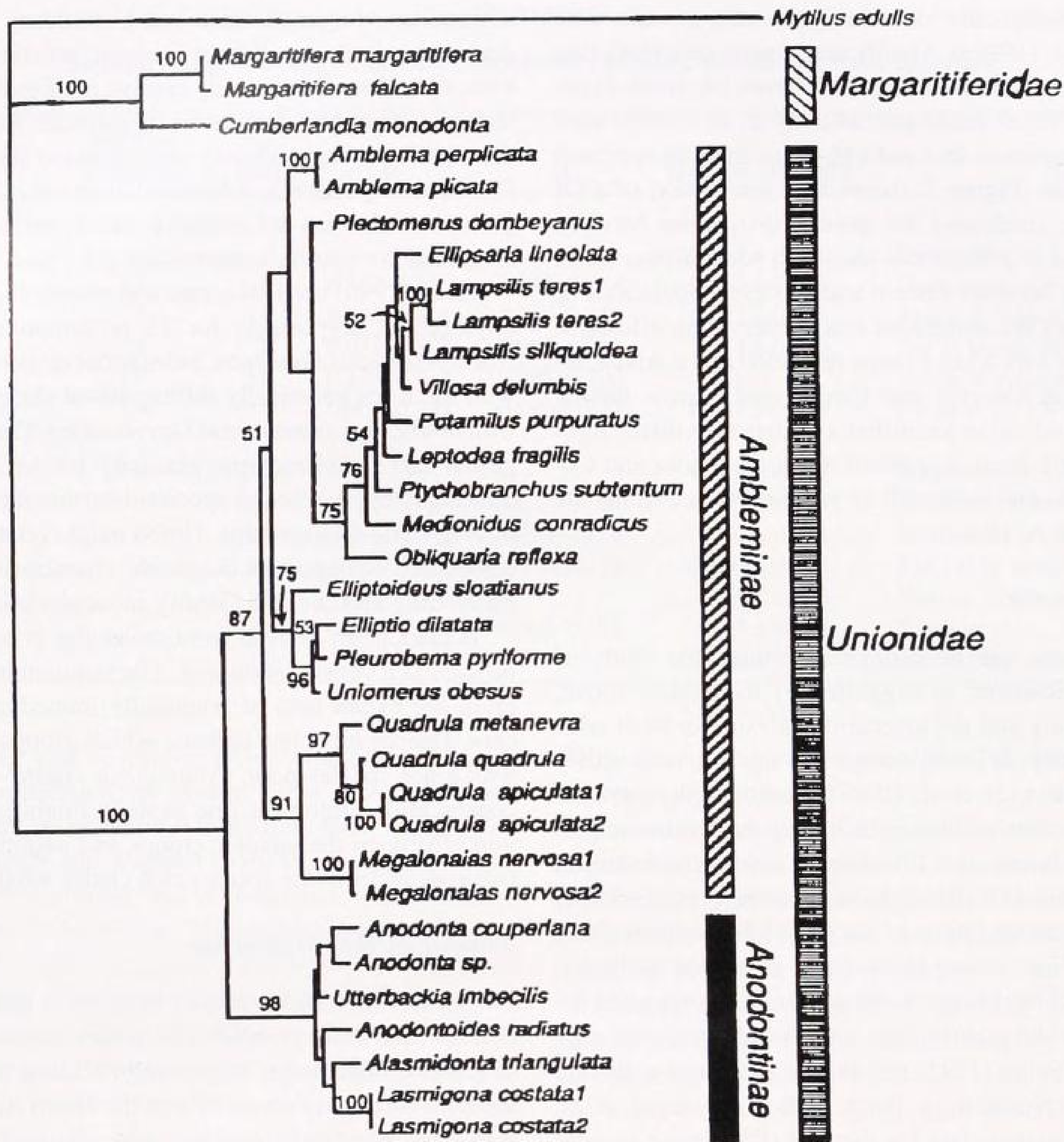

Figure 4. Phylogram generated from maximum parsimony analysis of 16S rRNA sequences for 29 species of North American unionaceans. (from Lydeard et al. 1996)

ability of success with in-situ preservation, translocation, or laboratory propagation would be greatly enhanced if reproductive requirements were known.

Their association with fish limits natural dispersal of unionids. The type and number of host fish utilized will affect dispersal and gene flow probabilities and, therefore, the likelihood of genetic divergence among populations. Kat (1984) argued that differences in patterns of genetic structure (determined with allozymes) in two widely distributed bivalves might be attributable to host fish relationships. *Elliptio complanata* commonly utilizes the yellow perch, *Perca flavescens,* as a host. Yellow perch are restricted to fresh or slightly brackish waters, display territorial behavior, and may be restricted to particular areas within a drainage. *Elliptio complanata* exhibited considerable differentiation in allozymes and morphological characteristics between drainages and even within drainages, as might be predicted from the limited dispersal associated with this host fish. In contrast, *Anodonta implicata* uses an anadromous host fish (alewife, *Alosa pseudoharengus*) which displays considerable movement within and, potentially, among drainages. *Anodonta implicata* populations exhibited relatively little genetic or morphological divergence among populations even at the extremes of their range.

Currently, most determinations of host fish suitability are done using laboratory exposures (Zale and Neves 1982). Other studies use morphological traits (e.g., glochidial shape, presence/absence of hooks) to identify glochidia collected from fish taken in the field. Morphological traits often provide identification only to subfamily. A method that would allow glochidia to be identified to species after removal from fish would provide much-needed data on this important portion of the life history and significantly improve our ability to manage this fauna. To be useful, such a technique must accommodate many taxa (e.g., the Cumberland drainage is home to 87 unionid species) and also be able to distinguish among co-occurring congeners (e.g., at least five species of *Elliptio* occur in the Savannah River).

White et al. (1994) reported a molecular genetic technique that has promise for identification of glochidia taken from infested fish. They used RFLP patterns (ITS1 region) of encysted glochidia taken from fish and matched them to RFLP patterns of adult unionids to make identifications. However, the discriminating power of RFLPs was not sufficient below the level of genus for the 25 unionid species found in their French Creek, Pennsylvania study area. The authors suggested that DNA sequence data should be able to distinguish species. DNA sequences are unique to species or populations and could be used in habitats where many freshwater bivalves and fish co-occur.

Hatchery Populations

The rapid decline of many freshwater bivalve populations has led to increased efforts to rear critically endangered species in

hatcheries for eventual release to the wild. One goal must be the maintenance of genetic characteristics that maximize the probability of success when bivalves are repatriated. In the hatchery situation, two issues of concern lend themselves to a genetic approach: changes in allele frequency and loss of genetic variation due to drift and domestication during captivity. Genetic diversity may decline rapidly in captive populations because of small numbers of breeding adults, founder effects, and selection for hatchery-adapted stocks. Allozyme or DNA-based genetic markers provide convenient methods to characterize stocks at their establishment in the hatchery and to monitor stock integrity over generations of maintenance in the hatchery. An additional application of molecular genetic markers is the identification of unique stocks. Where genetically distinct populations (ESUs) have been identified, it is desirable to maintain separate gene pools as these may represent locally adapted gene complexes and their integrity would enhance the probability of success during repatriation.

Forensic Applications

Molecular genetic techniques have considerable potential for use in conservation law enforcement. Forensic applications include the identification of bivalve material that finds its way to commercial uses (Baker et al. 1996). PCR techniques especially are well suited to this problem. Only small amounts of tissue are needed and the many markers that can be developed make identification and determination of specimen origin very likely. For commercially exploited species transported across jurisdictions, this approach can lead to effective legal intervention. Molecular genetic data are currently being used to investigate allegedly poached washboards, *Megalonaias nervosa* (C. Lydeard, pers. com. to MM).

SIGNIFICANCE

Molecular genetic approaches are not a panacea for the conservation of freshwater bivalves. They are not appropriate for every species or every question, however, for some controversial issues such as the establishment of ESUs, they provide a powerful tool. Knowledge of genetic population structure, evolutionary relationships, and taxonomic validity of names is essential in making appropriate recommendations for conservation of unionid diversity. Among the more than 300 species of North American freshwater bivalves, 31% are considered endangered, 14.5% threatened, and 24% of special concern (Williams et al. 1993). Therefore, the need to document patterns of genetic variation and species boundaries in unionids is urgent. On-going loss of suitable habitat and habitat fragmentation will likely accelerate the urgency. Studies of patterns and processes affecting genetic differentiation are critically needed to increase the effectiveness of conservation efforts directed toward freshwater bivalves.

In the face of huge ecological and demographic issues in the conservation of freshwater bivalves, does it make sense to spend limited resources on genetic studies? The answer is a resounding "yes." Although ecological and demographic considerations are essential, genetic data provide much to the decision-making process. Conservation goals for freshwater bivalves are twofold: to increase the likelihood of species survival, and to conserve ecological and evolutionary processes for the long term. Both goals necessarily require the maintenance of genetic diversity. However, a simple inventory or description of genetic characteristics is not sufficient to meet these goals. Freshwater bivalves exhibit differences in mode of reproduction (hermaphrodite versus gonochoristic), reproductive strategy (simple or elaborate packaging of larvae), and host specificity (narrow or wide). Therefore, genetic data must be coupled with knowledge of biogeography, life history, and ecological data to formulate management plans that meet conservation goals.

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