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MOLECULAR GENETIC IDENTIFICATION TOOLS FOR THE UNIONIDS OF FRENCH CREEK, PENNSYLVANIA

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ABSTRACT

A molecular genetic key to the unionids of French Creek, Pennsylvania, an Allegheny River tributary, is presented here. The key is an integral part of a new approach to identifying unionid glochidia larvae attached to host fishes in the drainage. Working with tissue from adult unionids, we used the polymerase chain reaction (PCR) followed by restriction enzyme digests to find species-specific genetic "fingerprints" for the 25 species in the drainage. We have demonstrated the utility of the key by using it to identify 70 glochidia attached to fishes collected in the French Creek drainage.

Key words: Unionoidea, glochidial identification, PCR, RFLP analysis, ITS regions.

INTRODUCTION

North America's freshwater mussels (Bivalvia: Unionoidea) are declining precipitously in richness and abundance (e.g., Dennis, 1987; Anderson et al., 1991; Nalepa et al., 1991; Williams et al., 1992, 1993). Sizable gaps in knowledge of unionid reproductive requirements hamper current preservation efforts. Information on the identities of the host fishes upon which unionid glochidia larvae are obligate parasites is especially inadequate. Traditional methods of gathering such data have a variety of drawbacks.

To date, lists of unionid host fishes have been derived primarily in two ways. The first, which has its roots in artificial propagation efforts (e.g., Lefevre & Curtis, 1910, 1912; Coker et al., 1921), involves inoculating putative hosts with glochidia taken from gravid females of the unionid species of interest. Fishes in aquaria that ultimately contain metamorphosed juveniles are considered suitable hosts (e.g., Zale & Neves, 1982; Waller & Holland-Bartels, 1988). Unsuitable hosts launch immune responses that thwart glochidial encystment, preventing further development and causing glochidia to be shed (Arey, 1923a, 1932).

As the completion of metamorphosis requires a week to several months of attachment (Zale & Neves, 1982), this approach is often time-consuming. It is also ill-suited to systems with large numbers of potential host fishes. Moreover, drawing inferences from inoculation studies can be complicated by the fact that "suitable" host fishes can apparently acquire immunity to glochidia with repeated exposure, the duration and species specificity of which are poorly established (Reuling, 1919; Arey, 1923b; Fuller, 1974). To obtain unambiguous results, it is often necessary to collect putative hosts from unionid-free streams or to inoculate naïve fishes bred and raised in the laboratory. Finally, while artificial inoculation methods are appropriate if laboratory propagation of unionids is the only goal, the results of such studies might be inapplicable to organisms in their natural environments. Such studies disregard microhabitat preferences and specialized morphologies and behaviors (e.g., the waving of fish-like mantle flaps by gravid female *Lampsilis* species; Ortmann, 1911; Kraemer, 1970) that might modulate unionid-fish interactions *in situ*.

To circumvent these problems, several investigators (e.g., Wiles, 1975; Stern & Felder, 1978) have attempted morphology-based identification of glochidia attached to fishes. Such determinations have thus far entailed identifying the glochidia using dissecting microscopes or compound light microscopes.

There are drawbacks to this approach as well. Glochidia are less than 1 mm in diameter. Encystment makes them difficult to observe and might influence their shapes in unpredictable ways (Wiles, 1975). Closely related spe-

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cies, such as *Villosa nebulosa* (Conrad) and *Villosa vanuxemensis vanuxemensis [vanuxemi]* (I. Lea) (Zale & Neves, 1982), are difficult to distinguish from each other and are easily misidentified. Hoggarth (1992) reported that glochidia photographed by Wiles (1975) and identified by the author as *Pyganodon [Anodonta] cataracta* (Say) were actually *Alasmidonta undulata* (Say). Clarke (1981, 1985), Rand & Wiles (1982), and Hoggarth (1988) demonstrated that scanning electron microscopy can be used to distinguish among glochidia taken from gravid females. Whether their techniques can be adapted for species-level identification of glochidia from host fishes remains to be investigated, however.

The objective of the research described herein was to develop a new method for identifying glochidia attached to fishes, a method that exploits genetic differences among unionid species. The method utilizes restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) products. In combination, PCR and RFLP analysis are useful for performing sensitive analyses of minute quantities of DNA (e.g., Whitmore et al., 1992; Simon et al., 1993), such as those present in single glochidia. In short, a diagnostic suite of restriction sites (or "genetic fingerprint") is sought for each unionid species in the drainage of interest. Glochidia on host fishes are then identified on the basis of the "fingerprints" they possess.

MATERIALS AND METHODS

Study Site

The aquatic system for which the glochidial identification method was developed is the French Creek drainage, in southwestern New York and northwestern Pennsylvania (Fig. 1). French Creek is a fourth-order tributary to the upper Allegheny River. It drains approximately 3,000 km^2. Twenty-five unionid species (C. Bier, pers. comm.) and 53 fish species (J. Stauffer, unpubl. data) have been collected from the French Creek drainage recently, making its fish and molluscan faunas the richest in Pennsylvania. Two of the drainage's unionid species, *Epioblasma torulosa rangiana* (I. Lea) and *Pleurobema clava* (Lamarck), are federally endangered and have no known hosts. Two additional species are considered globally threatened and seven are of special concern (Williams et al., 1993); of these nine, five have no known hosts.

LeBoeuf Creek is thought to harbor higher densities of *P. clava* than any other part of the drainage (A. Bogan, pers. comm.). To assess the utility of the identification technique, fishes were collected from LeBoeuf Creek at Moore Road bridge, just east of Route 19, 3 km south of LeBoeuf Gardens, Pennsylvania (Fig. 1). Full descriptions of the site and collection procedures are given by White (1994).

Specimen Collection and Preservation

Adult unionids. Adult unionids were collected throughout the French Creek drainage (Fig. 1, Table 1) in 1991, 1992, and 1993. Numbers of unionids collected ranged from one to 23 per species, with a median of six. Adult *Lasmigona costata* (Rafinesque), *Amblema plicata* (Say), and *Lampsilis siliquoidea* (Barnes) specimens were also collected from West Virginia (Dunkard Creek) and Ohio (lower Muskingum River, Little Muskingum River, and Big Darby Creek), so that their genetic "fingerprints" could be compared with those of French Creek specimens to evaluate the key's applicability to other drainages.

Adult unionids were collected using masks and snorkels or Plexiglas-bottomed buckets. Nonendangered species were transported to the laboratory either alive (wrapped in cheesecloth in chlorine-free ice water) or frozen on dry ice. In the laboratory, live unionids were either killed and frozen at −80°C, or maintained in aquaria in which currents were established. Two small (5- to 100-mg) pieces of foot tissue were excised from each individual in the laboratory using a sterile scalpel blade or scissors. Both samples were frozen at −80°C, one for nucleic acid extraction and the other for voucher material. The remaining tissue was preserved with the valves in 70% ethanol, also as voucher material. To facilitate future molecular genetic examination, the latter tissue was not fixed in formalin. All voucher material was deposited into the mollusc collection of the Academy of Natural Sciences in Philadelphia upon completion of the research (Dry Catalog # 398499-398500; Alcohol Catalog # A18354-A18438; Frozen Catalog # F100-F118).

For endangered unionids, a single tissue sample was obtained from each specimen at streamside by relaxing its adductor muscles in soda water and clipping off a 5- to 50-mg

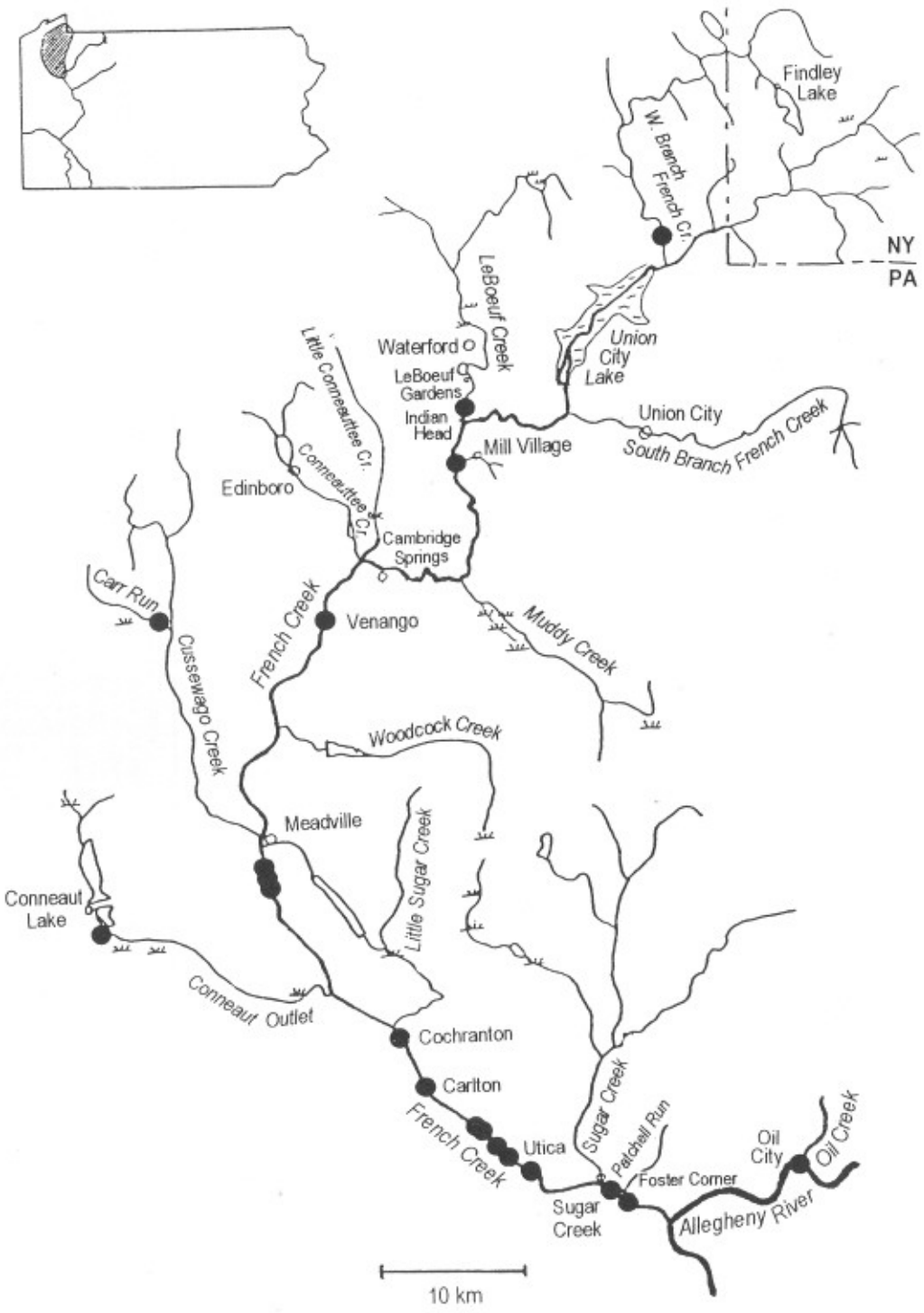


FIG. 1. Location of French Creek drainage collection sites. (For site descriptions, see Table 1 and White, 1994).

TABLE 1. Number of unionids collected, by species and site.

	Site																					
	French Creek drainage																	Dunkard Creek			Ohio		
Species	WB	LB	MV	VN	CR	MD	CO	CN	CA	U1	U2	U3	U4	U5	SC	FC	OC	MM	PN	BL	MU	LM	BD
Actinonaias ligamentina				4		8			1	2						3							
Alasmidonta marginata				4		3			1	1													
Amblema plicata									2	4						1		11	4		18		
Anodontoides ferussacianus	3																						
Elliptio dilatata				7						4			1				1						
Epioblasma torulosa				2									1	1									
Epioblasma triquetra			1	1								1											
Fusconaia subrotunda											5	1											
Lampsilis cardium				4+1?		1?					1+1?					1							
Lampsilis fasciola				1		1				1		1	1										
Lampsilis ovata				1+1?		1				2+1?	1?												
Lampsilis siliquoidea			1	3		1	1			1								5	2	10		5	1
Lasmigona complanata							1																
Lasmigona compressa	1				3																		
Lasmigona costata				9		7		1	2	1						1		22				2	6
Ligumia nasuta							11																
Ligumia recta				1					1	1							1						
Pleurobema clava		2					1																
Pleurobema sintoxia										1	3					1							
Ptychobranchus fasciolaris				6		1		1		3	1					1							
Pyganodon grandis			1				4																
Quadrula cylindrica				2						3	4												
Strophitus undulatus				10					5	3		4											
Villosa fabilis				1				1		11			3	1									
Villosa iris															6								

BD = Big Darby Cr., Ohio; BL = Dunkard Cr. at Blacksville, West Virginia; CA = French Cr. at Carlton; CN = French Cr. at Cochranton; CO = Conneaut Outlet; CR = Carr Run; FC = French Cr. at Foster Corner; LB = LeBoeuf Cr.; LM = Little Muskingum R., Ohio; MD = French Cr. downstream of Meadville; MM = Dunkard Cr. near Mt. Morris, Pennsylvania; MU = lower Muskingum R., Ohio; MV = French Cr. at Mill Village; OC = Allegheny River at Oil City; PN = Dunkard Cr. near Pentress, West Virginia; SC = French Cr. near town of Sugar Cr.; U1 = French Cr. 2.8 km upstr. of Utica; U2 = French Cr. 2.7 km upstr. of Utica; U3 = French Cr. 1.1 km upstr. of Utica; U4 = French Cr. at Utica; U5 = French Cr. 1 km downstr. of Utica; VN = French Cr. at Venango; WB = West Br. French Cr.; ? signifies specimen of questionable identity

piece of foot using a sterile scalpel blade or scissors (Pennsylvania Fish and Boat Commission permit number 142 (Type I); procedure reviewed prior to permitting by the United States Fish and Wildlife Service). Tissue samples were frozen immediately on dry ice for transportation to the laboratory, where they were kept at −80°C pending nucleic acid extraction. After a 10- to 15-min recovery period in a bucket of streamwater, the specimens were photographed and returned to natural positions in the substrate as close to their original locations as possible.

Fishes. Fishes were collected throughout French Creek by kick-seining and were transported to the laboratory on dry ice. In the laboratory, a 5- to 100-mg piece of muscle was excised from the body wall of each and was frozen at −80°C prior to nucleic acid extraction. The remainder of each specimen was also frozen at −80°C as voucher material.

Glochidia. Glochidia of known identity were obtained from marsupia of gravid nonendangered female unionids collected and frozen as described above. Glochidia of unknown identity were obtained from fishes collected throughout French Creek by kick-seining. The fishes were transported to the laboratory alive, maintained in an aquarium for one week, then killed and frozen at −80°C; unencysted glochidia were presumed to have been shed during the holding period. Encysted glochidia were removed as described below.

Laboratory Techniques

Nucleic acid extraction. For adult unionids, unattached glochidia, and fishes, a standard phenol-chloroform extraction protocol (after Kocher et al., 1989) was used to isolate total nucleic acids. Each tissue sample was minced over ice using a sterile scalpel blade, then transferred to a 1.5-ml microfuge tube and homogenized in 500–800 µl of extraction buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; 125 mM NaCl; 0.1% SDS; 50 mM DTT; 5 µg/µl proteinase K) using a flame-sealed 1000-µl pipette tip; different scalpel blades and pipette tips were used for each sample, to prevent cross-contamination. Homogenized samples were incubated 2–24 hrs at 37°C, then extracted sequentially with equal volumes of Tris-buffered phenol, 50% phenol-50% chloroform, and chloroform (= 24 chloroform: 1 isoamyl alcohol, v:v; Sambrook et al., 1989). Samples were centrifuged 4–5 min at 16,000 × g during each extraction to separate the phases. After the final extraction, 0.05 volume of 5 M ammonium acetate and two volumes of cold absolute ethanol were added to each sample. Samples were placed at −80°C for 15–30 min, then spun 15–45 min at 16,000 × g at 4°C. Supernatants were decanted and pellets were dried in a Savant SpeedVac Concentrator. Pellets were resuspended in 10–25 µl of sterile distilled water, depending on their size, and stored at −20°C. Even when no pellet was visible in a tube, 10 µl of sterile distilled water was added and the sample was stored at −20°C. Extractions were assayed on 0.8%-agarose minigels stained with ethidium bromide and were diluted 0–1000× depending upon estimated DNA concentration.

For glochidia attached to fishes, an extraction protocol similar to that described by Martin et al. (1992) for fish oocytes was used. Each glochidium was removed from its host over ice using sterile forceps and a dissecting light microscope, then transferred with a 200-µl pipette tip to a 1.5-ml microfuge tube containing 30 µl of buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1 µg/ml proteinase K; 1 µg/ml bovine serum albumin). Nonidet P-40 was added to a final concentration of 1%. Solutions were heated to 95°C for 5 min in a thermal cycler, diluted to a final volume of 50 µl with sterile distilled water, and stored at 4° or −20°C. Extractions were not assayed prior to amplification, as they contained too little DNA to be visualized with ethidium-bromide staining (data not shown).

Amplification. Reaction volumes of 50 or 100 µl were used. Reaction mixtures consisted of 0.5–2.0 µl of diluted template DNA; 1 µM of each primer (0.2 µM of each RAPD primer); 0.1 mM each of dATP, dCTP, dGTP, and dTTP; 2.0–2.5 units of Perkin-Elmer Cetus Taq polymerase; and manufacturer-supplied buffer at 1× final concentration (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 15 mM $MgCl_2$; 0.01% (w:v) gelatin). For glochidia from host fishes, 1–10 µl of undiluted template was used.

Primer sequences were as follows. ITS-1 of nuclear rDNA: 5′-TAACAAGGTTTCCGTAGGTG-3′ (18S region) and 5′-AGCTRGCTGCGTTCTTCATCGA-3′ (5.8S region); ITS-1 through ITS-2: 5′-TCCGTAGGTGAACCTGC-

GG-3′ (ITS1 of Lee & Taylor, 1992; 18S region) and 5′-TCCTCCGCTTATTGATATGC-3′ (ITS4 of Lee & Taylor, 1992; 28S region); 12S mitochondrial rDNA: 5′-TAATAATAAGAGCGACGGGCGATGTGT-3′ (adapted from H1478 of Kocher et al., 1989 using sequence data for *Drosophila yakuba* Burla (Clary & Wolstenholme, 1985)) and 5′-TAATAAAAAACTAGGATTAGATACCCTATTAT-3′ (adapted from L1091 of Kocher et al., 1989); RAPD primer A-02: (5′-TGCCGAGCTG-3′; Operon Technologies, Inc., Alameda, CA). Rationales for primer choices are discussed in White (1994) and White et al. (1994).

Thirty-four amplification cycles were performed (1 min at 93°C, 1 min at 50°C, and 2 min at 72°C) followed by one cycle with increased extension time (9 min). For RAPD PCR, 45 amplification cycles of 1-min denaturation at 94°C, 1-min reannealing at 36°C, and 2-min extension at 72°C were performed. Reaction products were assayed on 0.8–2.0% agarose minigels stained with ethidium bromide.

Restriction Enzyme Digestion. Restriction enzyme digests were performed in 10- to 20-µl reaction volumes consisting of 8–12 µl of PCR product, 5–15 units of restriction enzyme, and the manufacturer-supplied buffer at a final concentration of 1×. Digests were conducted at the manufacturer-recommended temperature (usually 37°C) for 4–48 hrs. Restriction fragments and uncut PCR products were assayed on 2.0%-agarose gels stained with ethidium bromide. Efforts to separate poorly-resolved fragments with 6–10% polyacrylamide or 2–4% MetaPhor high-resolution agarose met with limited success and were ultimately abandoned.

RESULTS

Key to the Unionids of French Creek

The following key was developed for identification of French Creek unionid glochidia. One proceeds through the key by amplifying the genomic region indicated in **bold text,** digesting the PCR product with the restriction enzyme listed after the *x,* and assigning a letter to the resulting restriction fragment pattern (by referring to the accompanying figure and/or to the fragment size data in Appendix 1). Assaying undigested PCR products alongside digested products facilitates pattern interpretation and is highly recommended. Superscripts refer to notes that follow the key. While the key likely reflects phylogeny to some extent, the data from which it was constructed are insufficient for testing specific hypotheses about relationships; thus, the key should be considered artificial.

1. **ITS-1** × MspI (Fig. 2)
   - A . . . . . 2[a]
   - B . . . . . *Ligumia nasuta* (Say)
   - C . . . . . 9[b]
   - D . . . . . *Amblema plicata* (Say)[c]
   - E . . . . . *Quadrula cylindrica* (Say)
   - F 12
   - G . . . . . *Strophitus undulatus* (Say)[d]
   - H . . . . . *Alasmidonta marginata* Say
2. **ITS-1** × Sau96I (Fig. 3)
   - A 3
   - B 8
3. **12S** × HaeIII (Fig. 4)
   - A 4
   - B . . . . *Actinonaias ligamentina* (Lamarck)
   - C . . . . . *Lampsilis siliquoidea* (Barnes)[e]
4. **ITS 1–2** × MspI[f] (Fig. 5)
   - A 5
   - A′ 7
   - B . . . . . *Lampsilis fasciola* Rafinesque
5. **12S** × RsaI (Fig. 6)
   - A . . . . . *Villosa iris* (I. Lea)
   - 0 . . . . . *Epioblasma* spp. . . . . 6[g]
6. **ITS 1–2** × MboII (Fig. 7)
   - A . . . . . : *Epioblasma torulosa rangiana* (I. Lea)[h]
   - A′ . . . . *Epioblasma triquetra* (Rafinesque)
7. **ITS-1** × AvaI (Fig. 8)
   - A . . . . . *Lampsilis cardium* Rafinesque, *Lampsilis ovata* (Say)[i]
   - 0 . . . . . *Ligumia recta* (Lamarck)
8. **ITS-1** × AccI (Fig. 9)
   - A . . . . . *Ptychobranchus fasciolaris* (Rafinesque)
   - 0 . . . . . *Villosa fabalis* (I. Lea)
9. **ITS-1** × BstEII (Fig. 10)
   - A . . . . . *Elliptio dilatata* (Rafinesque)
   - 0 10
10. **ITS 1–2** × MspI[f] (Fig. 11)
    - A 11
    - A′ . . . . . *Fusconaia subrotunda* (I. Lea)
11. **RAPD A-02** (Fig. 12)
    - A . . . . . *Pleurobema clava* (Lamarck)[h]
    - B . . . *Pleurobema sintoxia* [= *coccineum*] (Rafinesque)
12. **ITS-1** × BamHI (Fig. 13)
    - A . . . . . *Lasmigona costata* (Rafinesque)
    - 0 13

13. **ITS-1** × HinfI (Fig. 14)
  A . . . *Anodontoides ferussacianus* (I. Lea)
  B 14
14. **ITS-1** × MboII (Fig. 15)
  A . . . . . *Lasmigona compressa* (I. Lea)
  0 15
15. **ITS 1–2** × MspI[†] (Fig. 16)
  A . . . . . *Pyganodon* [= *Anodonta*] *grandis* (Say)
  A′ . . . . . *Lasmigona complanata* (Barnes)

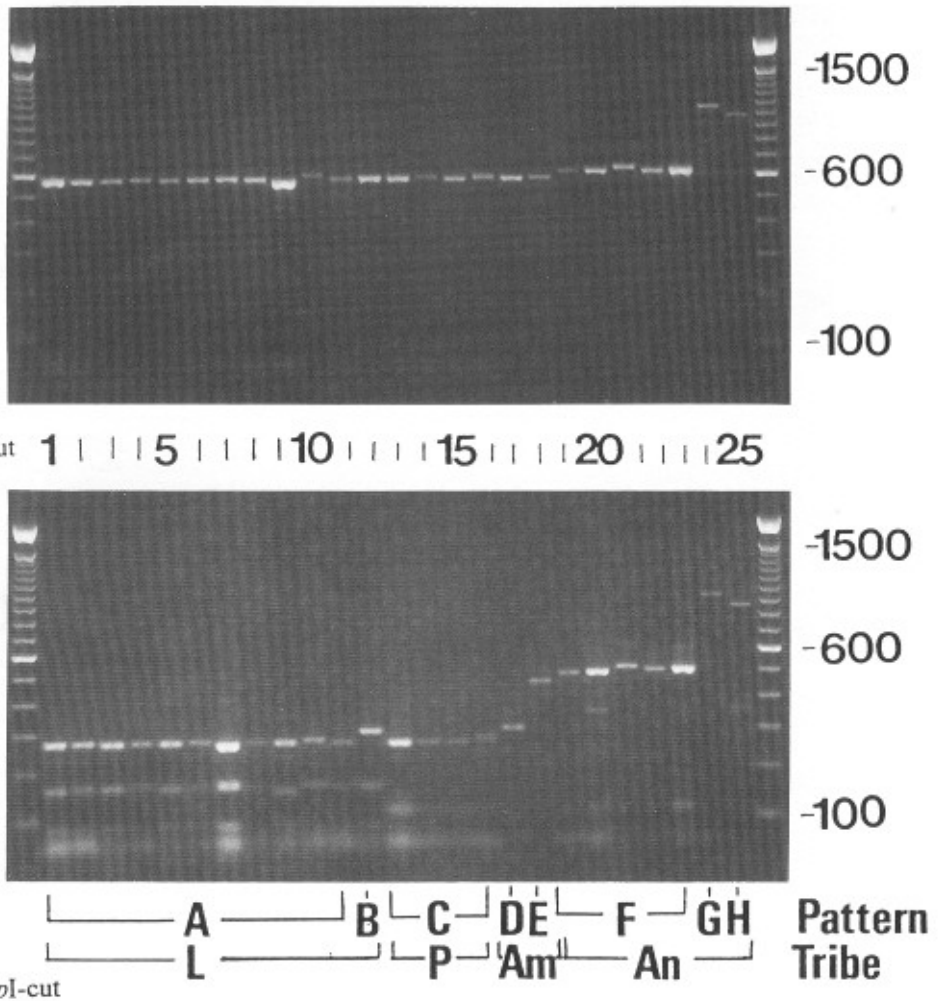
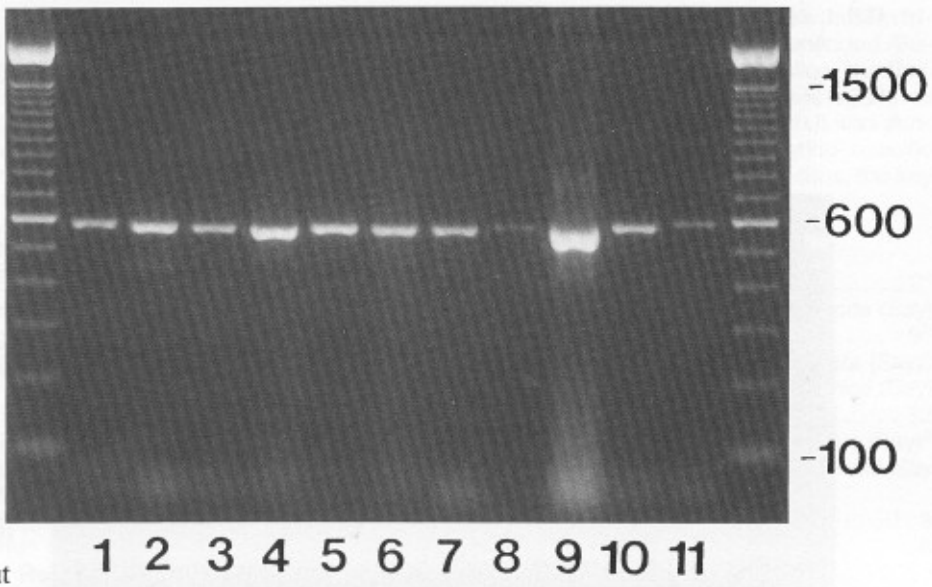
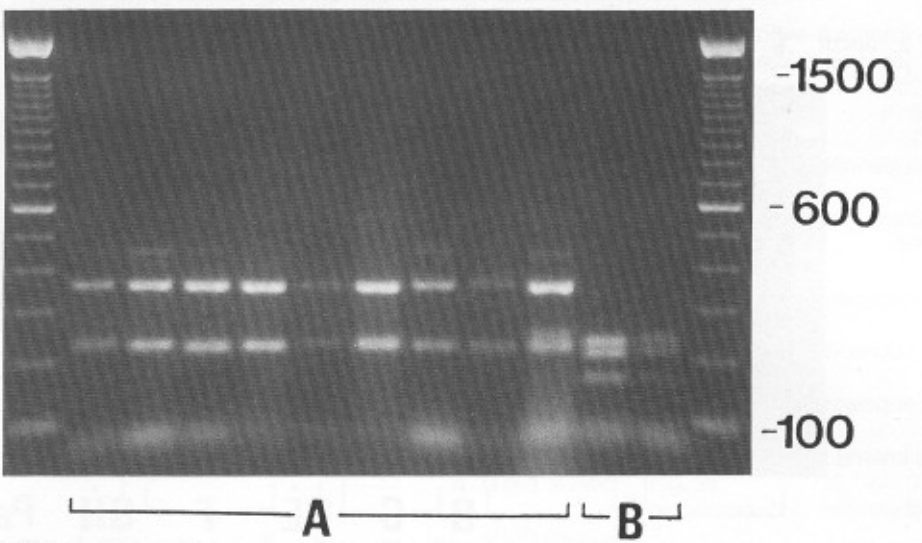


FIG. 2. ITS-1 PCR products from 25 French Creek unionid species digested with MspI. Restriction fragment patterns (A-H) separate species into their respective tribes. Tribe Am = Amblemini, An = (subfamily) Anodontinae, L = Lampsilini, P = Pleurobemini. Tribe Lampsilini (patterns A, B): 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *Lampsilis cardium*, 4 = *Lampsilis fasciola*, 5 = *Lampsilis ovata*, 6 = *Lampsilis siliquoidea*, 7 = *Villosa fabalis*, 8 = *Villosa iris*, 9 = *Actinonaias ligamentina*, 10 = *Ptychobranchus fasciolaris*, 11 = *Ligumia recta*, 12 = *Ligumia nasuta*; tribe Pleurobemini (pattern C): 13 = *Elliptio dilatata*, 14 = *Pleurobema clava*, 15 = *Pleurobema sintoxia*, 16 = *Fusconaia subrotunda*; tribe Amblemini (patterns D, E): 17 = *Amblema plicata*, 18 = *Quadrula cylindrica*; subfamily Anodontinae (patterns F, G, H): 19 = *Anodontoides ferussacianus*, 20 = *Pyganodon grandis*, 21 = *Lasmigona compressa*, 22 = *Lasmigona costata*, 23 = *Lasmigona complanata*, 24 = *Strophitus undulatus*, 25 = *Alasmidonta marginata*. Tribe designations follow Vaught (1989). Gels shown throughout key are 2.0% agarose. Size marker used throughout key is 100-bp ladder.

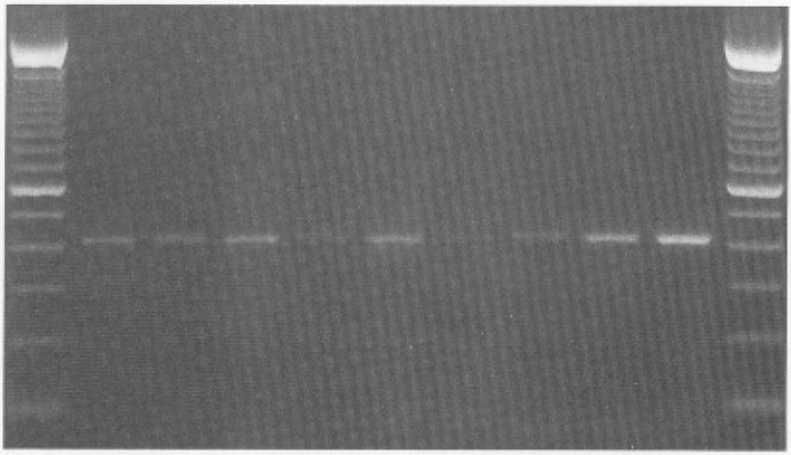


a. uncut

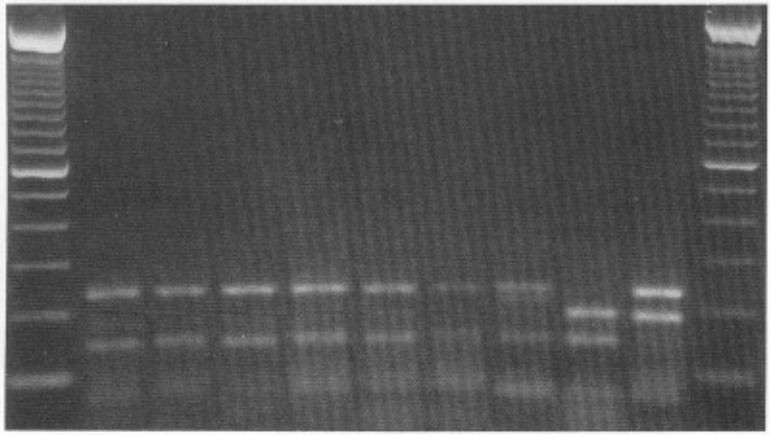


b. *Sau*96I-cut

FIG. 3. ITS-1 PCR products from "1-A" species digested with Sau96I. 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *Actinonaias ligamentina*, 4 = *Lampsilis cardium*, 5 = *Lampsilis fasciola*, 6 = *Lampsilis ovata*, 7 = *Lampsilis siliquoidea*, 8 = *Ligumia recta*, 9 = *Villosa iris*, 10 = *Villosa fabalis*, 11 = *Ptychobranchus fasciolaris*.



a. uncut 1 2 3 4 5 6 7 8 9



b. *Hae*III-cut A B C

FIG. 4. 12S PCR products from "2-A" species digested with HaeIII. 1 = *Lampsilis cardium*, 2 = *Lampsilis fasciola*, 3 = *Lampsilis ovata*, 4 = *Ligumia recta*, 5 = *Epioblasma torulosa rangiana*, 6 = *Epioblasma triquetra*, 7 = *Villosa iris*, 8 = *Actinonaias ligamentina*, 9 = *Lampsilis siliquoidea*.

Notes to Accompany the Key

[a]Includes *Ptychobranchus fasciolaris*, in contradiction to White et al., 1994; the specimen identified in White et al. (1994) as *P. fasciolaris* is almost certainly *Elliptio dilatata*.

[b]The *Pleurobema sintoxia* specimen from Foster Corner exhibited a unique pattern (Fig. 17).

[c]One of the 18 *Amblema plicata* specimens from the lower Muskingum River, Ohio, exhibited a unique pattern quite similar to that of *Ligumia nasuta* (Fig. 18).

[d]In contradiction to White et al., 1994; the specimen identified by White et al. (1994) as *Strophitus undulatus* was subsequently re-identified as *Pyganodon grandis* by A. E. Bogan.

[e]Two *Lampsilis siliquoidea* specimens from the French Creek drainage (one of the three

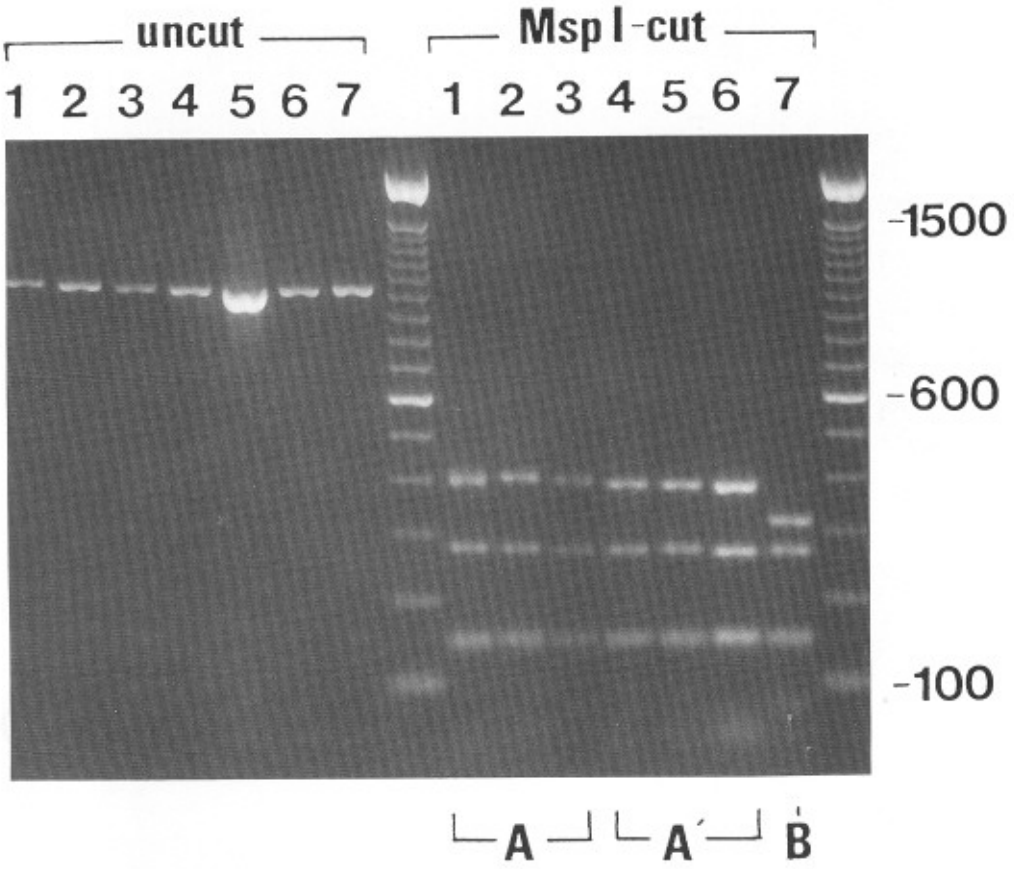


FIG. 5. ITS 1-2 PCR products from "3-A" species digested with MspI. 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *Villosa iris*, 4 = *Lampsilis cardium*, 5 = *Lampsilis ovata*, 6 = *Ligumia recta*, 7 = Lampsilis fasciola.

from Venango and the one from Conneaut Outlet) exhibited patterns with three bands instead of two (Fig. 19).

[f]A and A′ are most reliably distinguished by digesting samples of known DNA and assaying them in lanes adjacent to the unknown DNA. Digesting several samples of each known and unknown DNA is recommended, as it allows one to intersperse samples of each type on a single gel for easier detection of subtle length differences. Assays should be run on at least a 2%-agarose gel, for as long as possible, to achieve maximal separation.

[g]Couplet 6 reliably separates two of the four *Epioblasma torulosa rangiana* specimens examined (one of the two from Venango and the one from Utica) from the three *Epioblasma triquetra* specimens examined. The broader utility of this couplet is uncertain; it should be used with caution. Also see note f.

[h]federally endangered species

[i]*Lampsilis cardium* and *Lampsilis ovata* specimens could not be distinguished from each other using any of the primers and restriction enzymes tried (White, 1994: appendix B2). It is conceivable that these species hybridize in French Creek; some specimens exhibited intermediate shell morphologies and could not be identified to species with certainty on the basis of external characters (A. E. Bogan, pers. comm.).

Reliability of the Key

The key was tested extensively using adult unionids identified morphologically. In its annotated form, it proved valid for all French

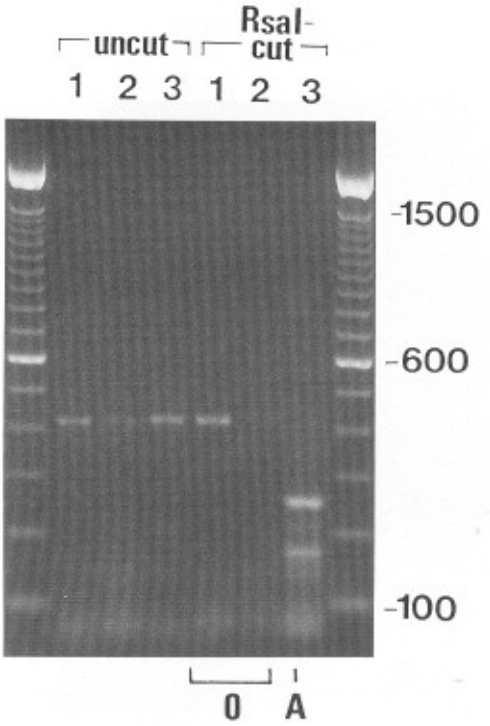


FIG. 6. 12S PCR products from "4-A" species digested with RsaI. 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *Villosa iris*.

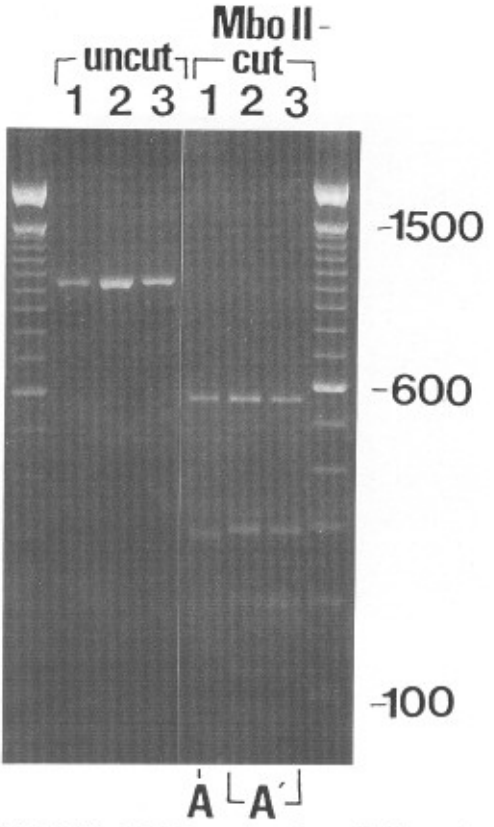


FIG. 7. ITS 1-2 PCR products from "5-0" species digested with MboII. 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *E. triquetra*.

Creek specimens examined. It was also valid for all Ohio and West Virginia *A. plicata* (Fig. 20), *L. siliquoidea*, and *L. costata* specimens examined. Moreover, glochidia obtained from a gravid French Creek female *L. costata* followed the key, exhibiting restriction fragment patterns identical to those of adult *L. costata* specimens, as expected (data not shown).

Identification of Unknown Glochidia with the Key

Four unknown glochidia from the gills of a tippecanoe darter (*Etheostoma tippecanoe* Jordan & Evermann) collected 20 July 1993 in French Creek downstream of Utica, Pennsylvania, exhibited restriction fragment patterns identical to those of adult *V. fabalis* specimens (unpubl. data). In a larger-scale test of the technique's utility, all glochidia found on fishes collected 6 June 1994 at the LeBoeuf Creek site were analyzed. Of the 115 glochidia

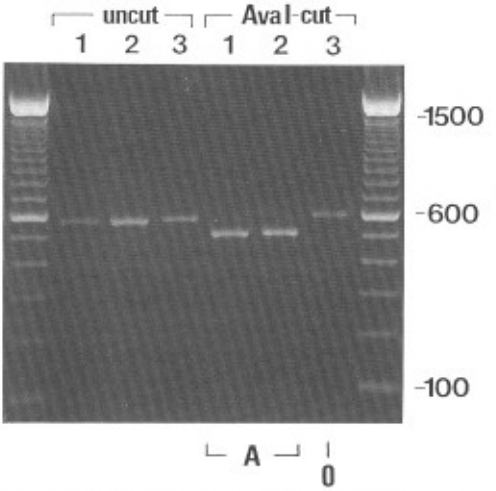


FIG. 8. ITS-1 PCR products from "4-A"' species digested with AvaI. 1 = *Lampsilis cardium*, 2 = *Lampsilis ovata*, 3 = *Ligumia recta*.

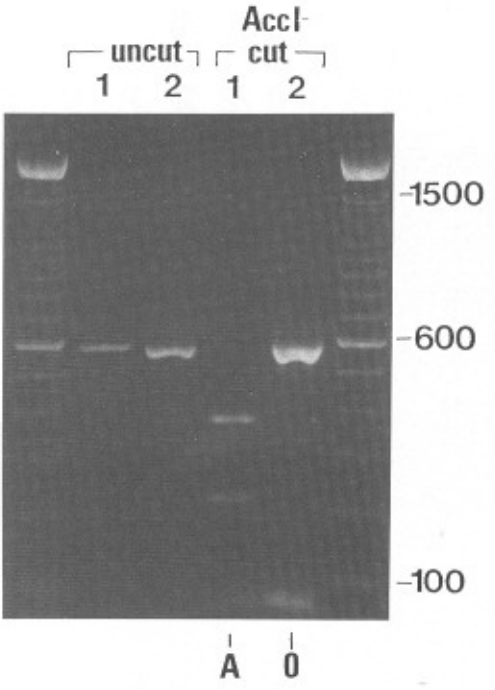


FIG. 9. ITS-1 PCR products from "2-B" species digested with AccI. 1 = *Ptychobranchus fasciolaris*, 2 = *Villosa fabalis*.

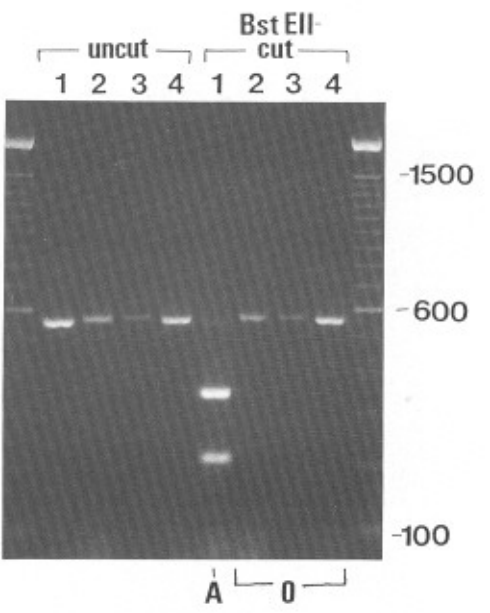


FIG. 10. ITS-1PCR products from "1-C" species digested with BstEII. 1 = *Elliptio dilatata*, 2 = *Fusconaia subrotunda*, 3 = *Pleurobema clava*, 4 = *Pleurobema sintoxia*.

processed, 72 (63%) were amplified successfully (i.e., their ITS-1 PCR products were visible on an agarose gel stained with ethidium bromide). Of these, 66 (92%) were identifiable; the other six yielded restriction fragments too faint to be seen. Fourteen of the 43 glochidia not amplified successfully were in the first set of samples, extracted using a protocol that differed slightly from that ultimately adopted. Disregarding this flawed first attempt, the amplification success rate was 72 out of 102 (71%).

All 66 glochidia identified exhibited the restriction fragment patterns characteristic of *Ptychobranchus fasciolaris* (Fig. 21), a species for which no hosts are currently known (Hoggarth, 1992). Four *Etheostoma blennioides* Rafinesque, three *Etheostoma flabellare* Rafinesque, five *Etheostoma nigrum* Rafinesque, and one *Etheostoma zonale* (Cope) harbored the glochidia. These four darter species are therefore suggested tentatively to be *P. fasciolaris* hosts, pending verification through laboratory inoculation studies.

DISCUSSION

Distinguishing Unionid DNA from Host Fish DNA

Without exception, the ITS-1 regions of the fishes examined are markedly different in length from those of the unionids. For single individuals of five of the six darter species examined (*E. blennioides, E. flabellare, E. tippecanoe, Etheostoma variatum* Kirtland, and *E. zonale*), the product is approximately 690–710 bp; for the sixth darter, *Etheostoma maculatum* Kirtland, the product is approximately 410 bp long (White, 1994: Fig. 2.3). Among most of the unionids, the ITS-1 product ranges from approximately 580 to 625 bp; for *Alasmidonta marginata* Say and *Strophitus undulatus* (Say), it is approximately 950–1,050 bp long (see uncut products in Fig. 2). Because the length ranges for fishes and unionids are non-overlapping, any host-fish DNA contaminating glochidial DNA is easily recognized as such. Furthermore, when ITS-1 PCR products of the six darter specimens are digested with MspI, they yield restriction fragment patterns different from all unionid patterns. Hence, even if the glochid-

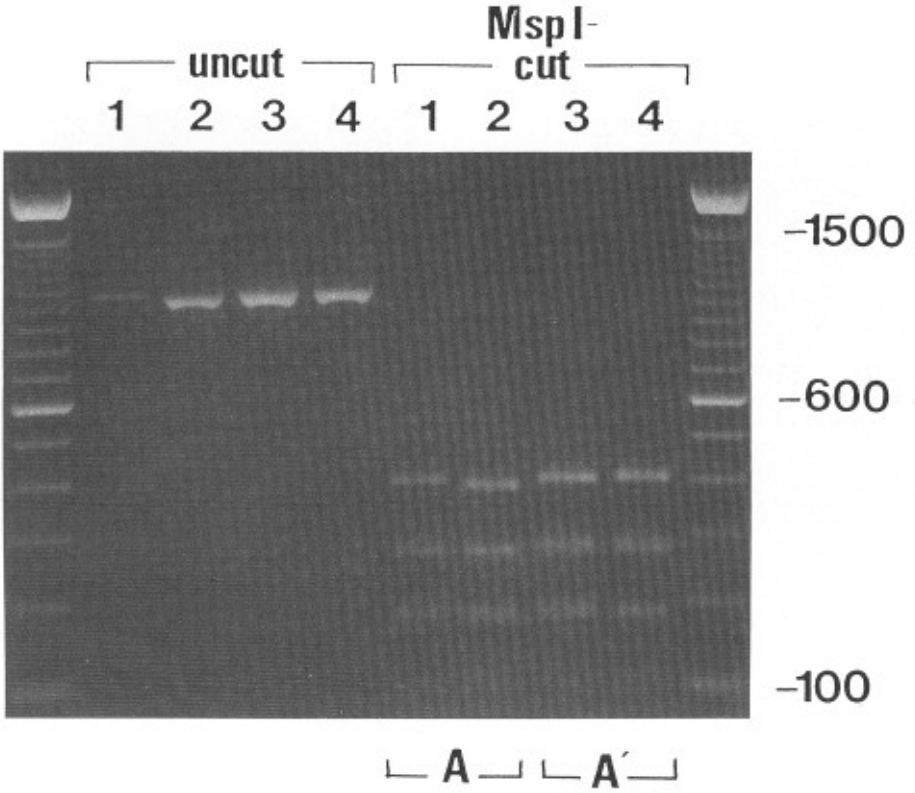


FIG. 11. ITS 1-2 PCR products from "9-0" species digested with MspI. 1 = *Pleurobema clava*, 2 = *Pleurobema sintoxia*, 3 = *Fusconaia subrotunda*, 4 = *F. subrotunda*.

ial identification method described herein were applied to fishes (e.g., salmonids) whose ITS-1 regions are close to the unionids' in length (Pleyte et al., 1992), contamination could be detected reliably by digesting the host fish's ITS-1 product and assaying it alongside the digested products of the glochidia it harbored. The contaminating DNA could be factored out of the RFLP analyses by disregarding restriction fragments present in both gel lanes.

Current Limitations of the Technique

Identifying glochidia on naturally infected fishes is a hit-or-miss approach to discovering hosts of a particular unionid species of interest. To maximize the chances of succeeding, it is important to collect fishes from sites where the unionid species of interest is abundant relative to other species (or at least where it is abundant relative to other sites). As the preliminary LeBoeuf Creek study

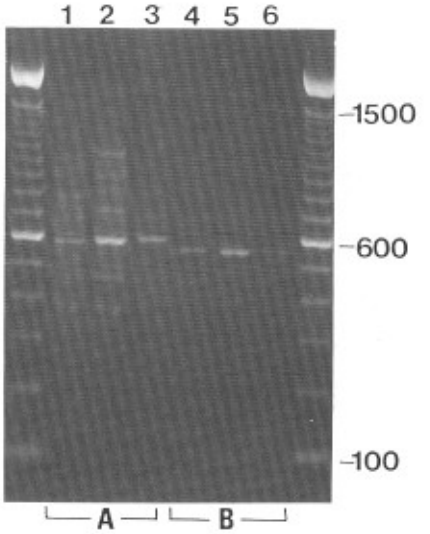


FIG. 12. RAPD A-02 PCR products from "10-A" species. 1–3 = *Pleurobema clava*, 4–6 = *Pleurobema sintoxia*.

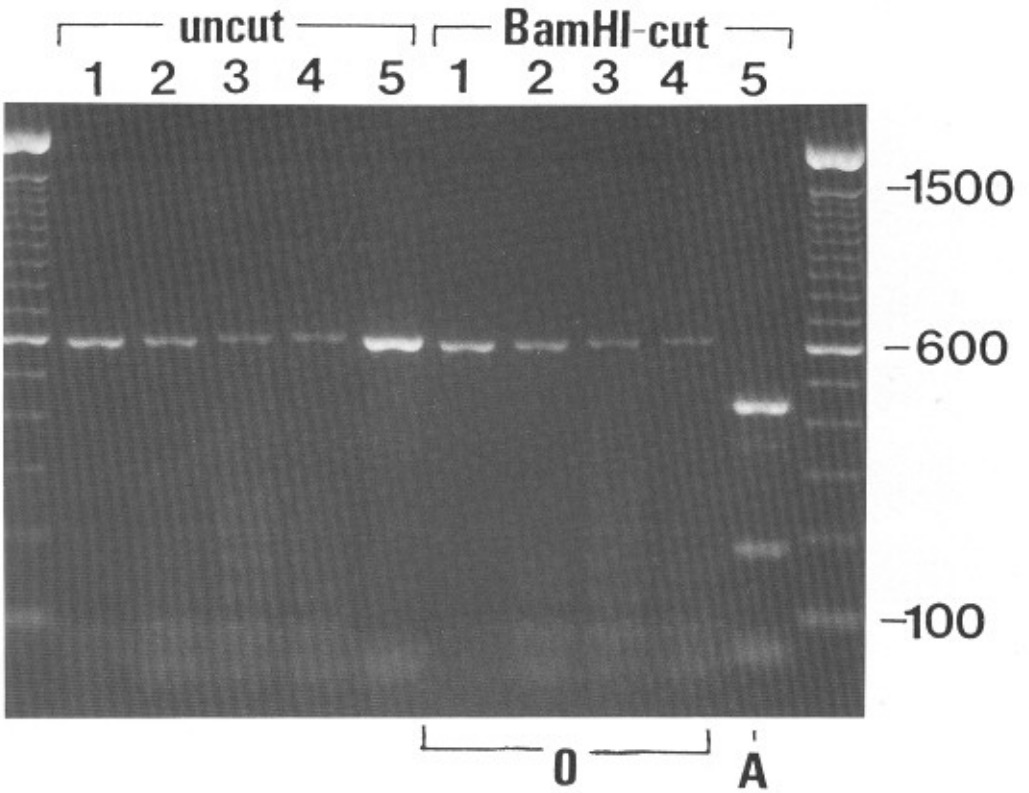


FIG. 13. ITS-1 PCR products from "1-F" species digested with BamHI. 1 = *Anodontoides ferussacianus*, 2 = *Pyganodon grandis*, 3 = *Lasmigona complanata*, 4 = *Lasmigona compressa*, 5 = *Lasmigona costata*.

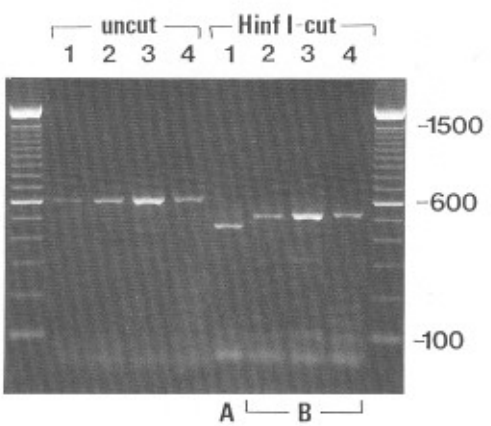


FIG. 14. ITS-1 PCR products from "12-0" species digested with HinfI. 1 = *Anodontoides ferussacianus*, 2 = *Pyganodon grandis*, 3 = *Lasmigona complanata*, 4 = *Lasmigona compressa*.

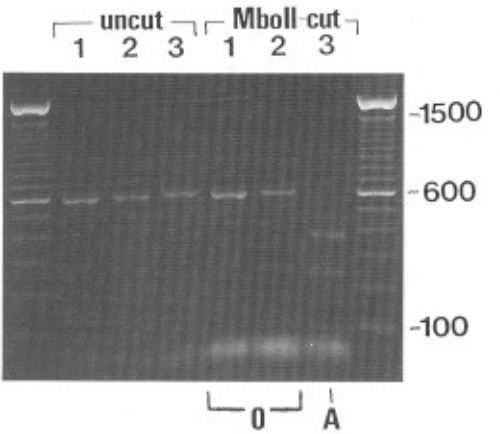


FIG. 15. ITS-1 PCR products from "13-B" species digested with MboII. 1 = *Pyganodon grandis*, 2 = *Lasmigona complanata*, 3 = *Lasmigona compressa*.

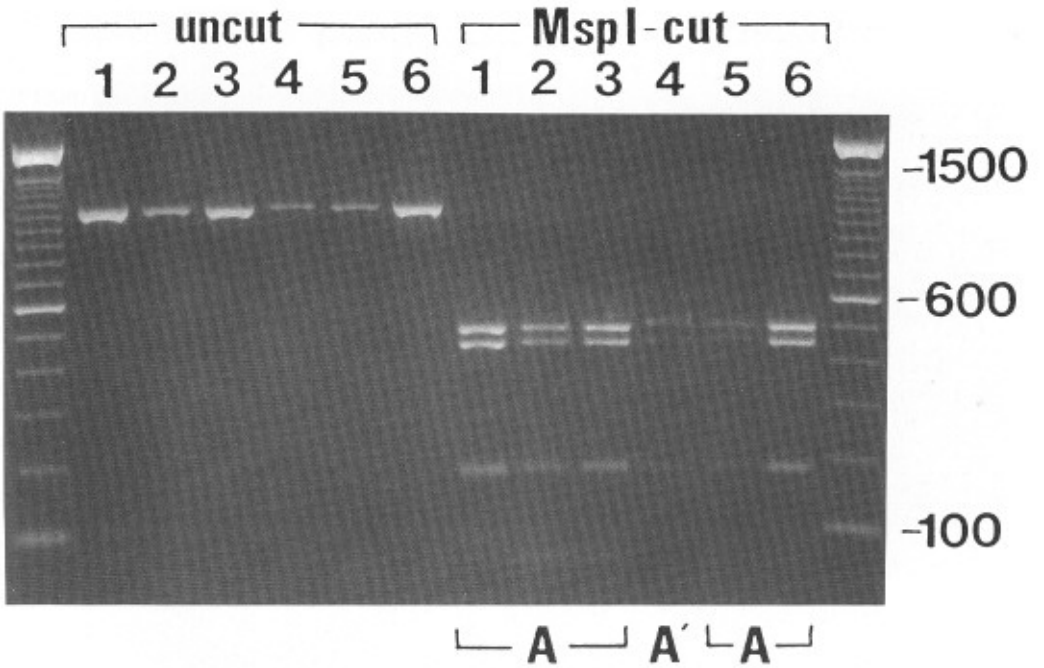


FIG. 16. ITS 1-2 PCR products from "14-0" species digested with MspI. 1–3, 5, 6 = *Pyganodon grandis;* 4 = *Lasmigona complanata.*

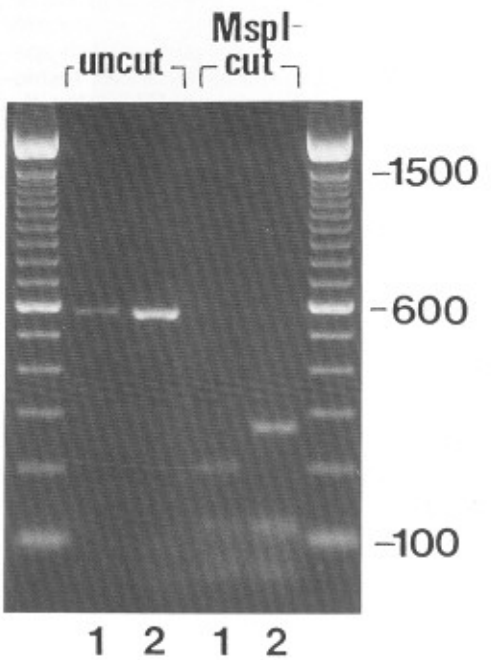


FIG. 17. ITS-1 PCR products from anomalous and standard *Pleurobema sintoxia* specimens digested with MspI. 1 = anomalous pattern, 2 = standard pattern.

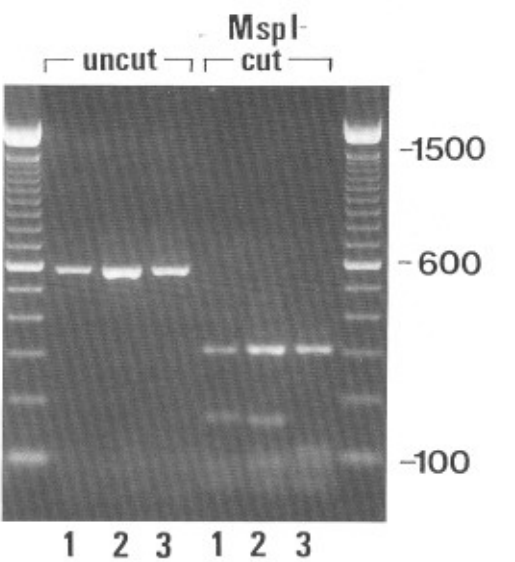


FIG. 18. ITS-1 PCR products from anomalous and standard *Amblema plicata* specimens digested with MspI. 1 = *Ligumia nasuta,* 2 = anomalous *Amblema plicata,* 3 = standard *A. plicata.*

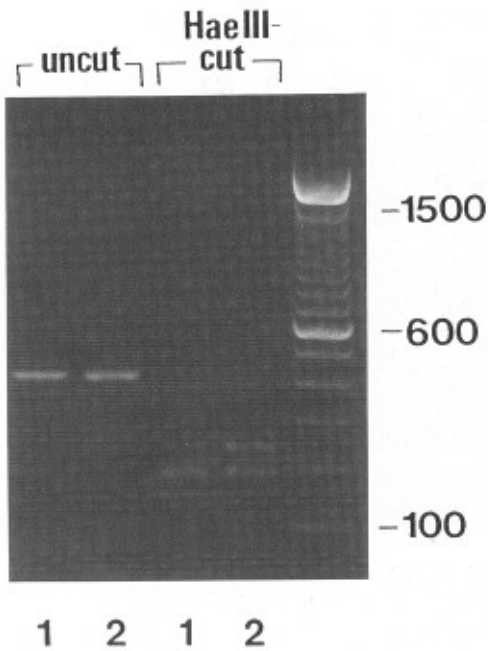


FIG. 19. 12S PCR products from anomalous *Lampsilis siliquoidea* specimens digested with HaeIII. Specimens exhibit 3-banded pattern instead of standard 2-banded pattern.

demonstrated, this will not guarantee success, however. Additionally, fishes should be collected repeatedly throughout the full duration of the unionid's breeding period.

The glochidial amplification procedure currently has a success rate below 100%. Most unsuccessful amplification attempts were likely the result of glochidia being lost during transfer from host to extraction buffer; once excised from the host, glochidia are extremely difficult to see. Improvements in the transfer technique could increase the amplification success rate dramatically. The identification success rate, already quite high, could probably be increased by gel-purifying and reamplifying very faint PCR products prior to restriction enzyme digestion.

Extending the Key Beyond French Creek

To apply the method to an aquatic system other than the French Creek drainage, some preliminary work is required. First, tissue samples must be obtained from several individuals of each unionid species found in the study system. Ideally, each species should be represented by specimens collected at a variety of sites.

Next, the reliability of the key, for the study-system species included in it must be assessed. All specimens of each such species should be analyzed using the key, to see whether they yield the expected restriction fragment patterns for each enzyme (as did the West Virginia and Ohio specimens we examined). If they do not, the key will have to be modified accordingly.

The key will also have to be extended to include any study-system species not found in French Creek. This is most easily accomplished as follows: first, analyze a single specimen of each new species, using the French Creek key. If a specimen yields a novel restriction fragment pattern for a certain couplet, test all individuals of the species to see if they share the pattern; if they do, modify the key accordingly. If a specimen yields no novel patterns, proving indistinguishable from a species already included in the key (or from another new species), screen single individuals of the indistinguishable species pair (or group) with a variety of primers and restriction enzymes until a diagnostic difference is found. (Consulting Appendix B of White, 1994, might prove useful in this regard.) Alternately, sequence a moderately variable region of the genome of each species and scan the sequence data for restriction site differences. Finally, verify that the differences found apply to all individuals of the species, then modify the key accordingly. Publish modified versions of the key promptly to save other investigators precious time and resources.

Overall Assessment of the Technique

Using a molecular genetic key to identify glochidia attached to fishes has distinct advantages over traditional means of identifying putative unionid hosts (White et al., 1994). The laboratory procedures are relatively fast and easy to perform. Once a key has been developed, glochidia can be identified in one or two days; the techniques involved can be learned (if not mastered) in a week. The method is also relatively inexpensive, particularly if one has access to a laboratory already equipped for molecular genetic research (see White, 1994: appendix C, for cost analysis).

The data generated to develop keys are potentially valuable to unionid systematists,

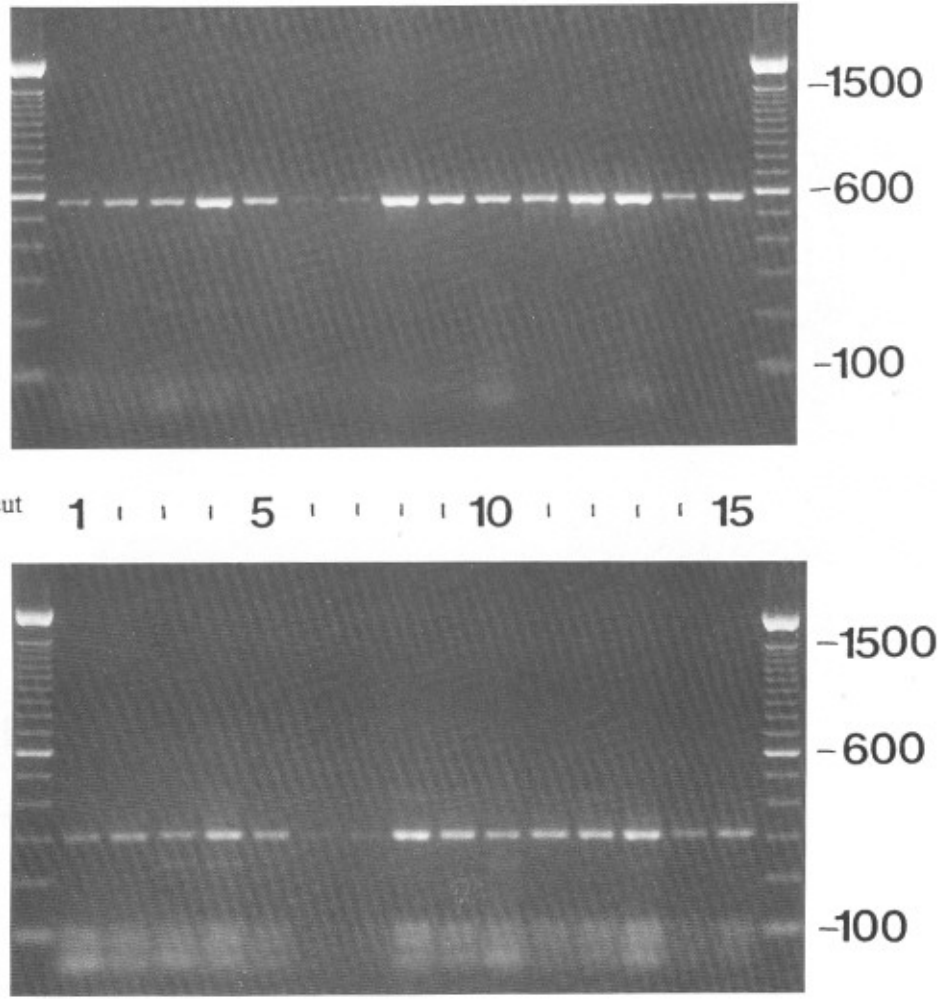


FIG. 20. ITS-1 PCR products from 15 *Amblema plicata* specimens from three drainages, digested with MspI. 1–5 = French Creek specimens, 6–10 = Dunkard Creek specimens, 11–15 = Muskingum River specimens.

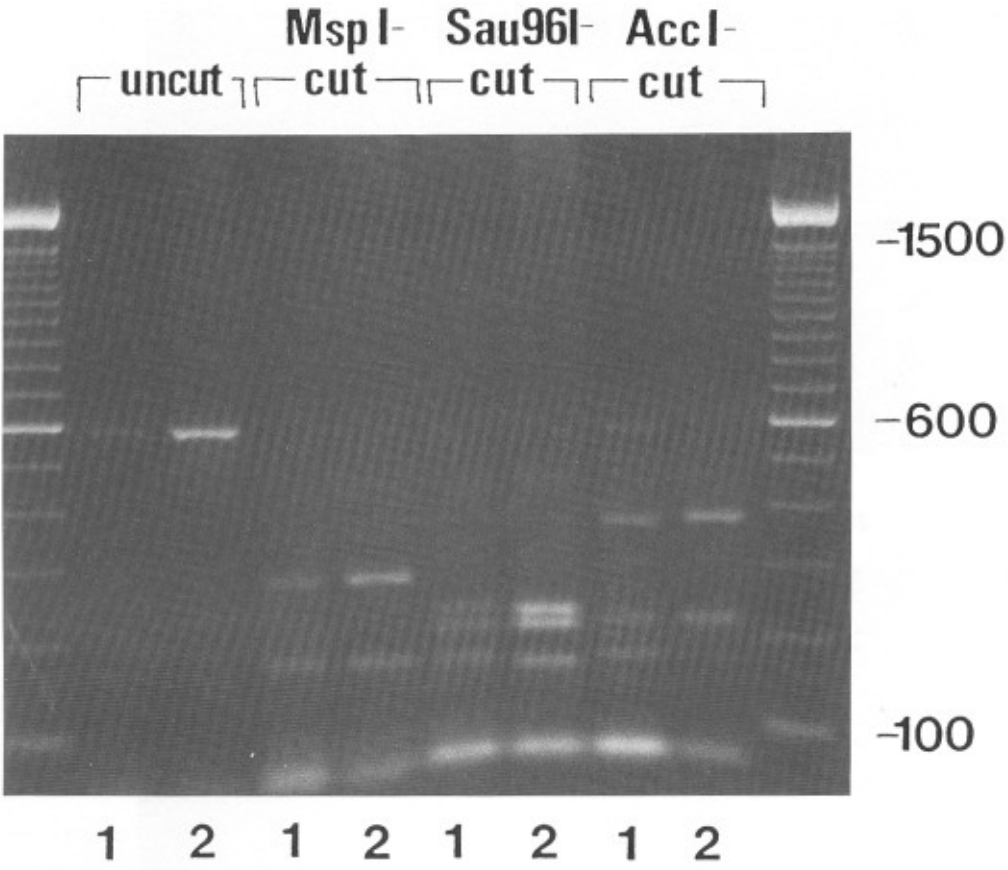


FIG. 21. ITS-1 PCR products from LeBoeuf Creek glochidium and adult *Ptychobranchus fasciolaris*, digested with MspI, Sau96I, and AccI. The glochidium exhibits restriction fragment patterns identical to those of the adult *P. fasciolaris*. The glochidium was removed from the gills of an *Etheostoma flabellare* specimen. 1 = glochidium, 2 = adult *P. fasciolaris*.

as well. For example, the RFLP analysis of ITS-1 shown in Figure 2 suggests that patterns of site gain and loss could demarcate tribal boundaries. In many organisms, this sort of information has been used to reconstruct phylogenetic relationships (reviewed in Avise, 1994). Our study was not designed to provide the complete matrix necessary to analyze this question, but our data (summarized in Appendix 2) do provide a starting point for systematists wishing to pursue the issue of higher relationships. (Note that many of the results presented in Appendix 2 are unreplicated and/or based on small numbers of specimens.)

The method is well suited to conservation work. It does not entail killing adult unionids and hence can be used with endangered species. It yields results that are relevant to natural communities. It can even furnish insights into subtle ecological matters, such as patterns of host-fish partitioning among unionids. Finally, it can be applied to diverse systems with large numbers of fish and unionid species.

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LITERATURE CITED

ANDERSON, R. M., J. B. LAYZER & M. E. GORDON, 1991, Recent catastrophic decline of mussels (Bivalvia: Unionidae) in the Little South Fork Cumberland River, Kentucky. *Brimleyana,* 17: 1–8.

AREY, L. B., 1923a, A microscopical study of glochidial immunity. *The Anatomical Record,* 26: 350.

AREY, L. B., 1923b, Observations on an acquired immunity to a metazoan parasite. *Journal of Experimental Zoology,* 38: 377–381.

AREY, L. B., 1932, A microscopical study of glochidial immunity. *Journal of Morphology,* 53: 367–379.

AVISE, J. C., 1994, *Molecular markers, natural history and evolution.* Chapman and Hall, New York, 511 pp.

CLARKE, A. H., 1981, *The freshwater molluscs of Canada.* National Museum of Natural Sciences, National Museums of Canada, Ottawa, Canada, 446 pp.

CLARKE, A. H., 1985, The tribe Alasmidontini (Unionidae: Anodontinae), Part II: *Lasmigona* and *Simpsonaias. Smithsonian Contributions to Zoology,* 399: 1–75.

CLARY, D. O. & D. R. WOLSTENHOLME, 1985, The mitochondrial DNA molecule of *Drosophila yakuba:* nucleotide sequence, gene organization, and genetic code. *Journal of Molecular Evolution,* 22: 252–271.

COKER, R. E., A. F. SHIRA, H. W. CLARK & A. D. HOWARD, 1921, Natural history and propagation of fresh-water mussels. *Bulletin of the United States Bureau of Fisheries,* 37: 77–181.

DENNIS, S. D., 1987, An unexpected decline in populations of the freshwater mussel, *Dysnomia* (= *Epioblasma*) *capsaeformis,* in the Clinch River of Virginia and Tennessee. *Virginia Journal of Science,* 38: 281–288.

FULLER, S. L. H., 1974, Clams and mussels (Mollusca: Bivalvia). Chapter 8, pp. 215–273, in: C. W. HART, JR. & S. L. H. FULLER, eds., *Pollution ecology of freshwater invertebrates.* Academic Press, New York, 389 pp.

HOGGARTH, M. A., 1988, *The use of glochidia in the systematics of the Unionidae (Mollusca: Bivalvia).* Doctoral Dissertation, The Ohio State University, 340 pp.

HOGGARTH, M. A., 1992, An examination of the glochidia-host relationships reported in the literature for North American species of Unionacea (Mollusca: Bivalvia). *Malacology Data Net,* 3(1–4): 1–30.

KOCHER, T. D., W. K. THOMAS, A. MEYER, S. V. EDWARDS, S. PÄÄBO, F. X. VILLABLANCA & A. C. WILSON, 1989, Dynamics of mitochondrial evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the Academy of Natural Sciences of the United States of America,* 86: 6196–6200.

KRAEMER, L. R., 1970, The mantle flap in three species of *Lampsilis* (Pelecypoda: Unionidae). *Malacologia,* 10: 225–282.

LEE, S. B. & J. W. TAYLOR, 1992, Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molecular Biology and Evolution,* 9: 636–653.

LEFEVRE, G. & W. C. CURTIS, 1910, Experiments in the artificial propagation of freshwater mussels. *Bulletin of the United States Bureau of Fisheries,* 28: 615–626.

LEFEVRE, G. & W. C. CURTIS, 1912, Studies on the reproduction and artificial propagation of freshwater mussels. *Bulletin of the United States Bureau of Fisheries,* 30: 105–201.

MARTIN, A. P., R. HUMPHREYS & S. R. PALUMBI, 1992, Population genetic structure of the armorhead, *Pseudopentaceros wheeleri,* in the North Pacific Ocean: application of the polymerase chain reaction to fisheries problems. *Canadian Journal of Fisheries and Aquatic Sciences,* 49: 2386–2391.

NALEPA, T. F., B. A. MANNY, J. C. ROTH, S. C. MOZLEY & D. W. SCHLOESSER, 1991, Long-term decline in freshwater mussels (Bivalvia: Unionidae) of the western basin of Lake Erie. *Journal of Great Lakes Research,* 17: 214–219.

ORTMANN, A. E., 1911, A monograph of the najades of Pennsylvania. pp. 279–347, In: W. J. HOLLAND, ed., *Memoirs of the Carnegie Museum,* 4(6). Carnegie Institute, Pittsburgh, Pennsylvania.

PLEYTE, K. A., S. D. DUNCAN & R. B. PHILLIPS, 1992, Evolutionary relationships of the salmonid

fish genus *Salvelinus* inferred from DNA sequences of the first internal transcribed spacer (ITS 1) of ribosomal DNA. *Molecular Phylogenetics and Evolution,* 1: 223–230.

RAND, T. G. & M. WILES, 1982, Species differentiation of the glochidia of *Anodonta cataracta* Say, 1817 and *Anodonta implicata* Say, 1829 (Mollusca: Unionidae) by scanning electron microscopy. *Canadian Journal of Zoology,* 60: 1722–1727.

REULING, F. H., 1919, Acquired immunity to an animal parasite. *Journal of Infectious Diseases,* 24: 337–346.

SAMBROOK, J., E. F. FRITSCH & T. MANIATIS, 1989, *Molecular cloning: a laboratory manual,* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 3 vol.

SIMON, C., C. MC INTOSH & J. DENIEGA, 1993, Standard restriction fragment length analysis of the mitochondrial genome is not sensitive enough for phylogenetic analysis or identification of 17-year periodical cicada broods (Hemiptera: Cicadidae): the potential for a new technique. *Annals of the Entomological Society of America,* 86: 228–238.

STERN, E. M. & D. L. FELDER, 1978, Identification of host fishes for four species of freshwater mussels (Bivalvia: Unionidae). *American Midland Naturalist,* 100: 233–236.

VAUGHT, K. C., 1989, *A classification of the living Mollusca.* American Malacologists, Inc., Melbourne, Florida, 195 pp.

WALLER, D. L. & L. E. HOLLAND-BARTELS, 1988, Fish hosts for glochidia of the endangered freshwater mussel *Lampsilis higginsi* Lea (Bivalvia: Unionidae). *Malacological Review,* 21: 119–122.

WHITE, L. R., 1994, *Identification of freshwater mussel glochidia on host fishes from French Creek, Pennsylvania, using molecular techniques.* Doctoral dissertation, The Pennsylvania State University, 75 pp.

WHITE, L. R., B. A. MC PHERON & J. R. STAUFFER, JR., 1994, Identification of freshwater mussel glochidia on host fishes using restriction fragment length polymorphisms. *Molecular Ecology,* 3: 183–185.

WHITMORE, D. H., T. H. THAI & C. M. CRAFT, 1992, Gene amplification permits minimally invasive analysis of fish mitochondrial DNA. *Transactions of the American Fisheries Society,* 121: 170–177.

WILES, M., 1975, The glochidia of certain Unionidae (Mollusca) in Nova Scotia and their fish hosts. *Canadian Journal of Zoology,* 53: 33–41.

WILLIAMS, J. D., S. L. H. FULLER & R. GRACE, 1992, Effects of impoundment on freshwater mussels (Mollusca: Bivalvia: Unionidae) in the main channel of the Black Warrior and Tombigbee rivers in western Alabama. *Bulletin of the Alabama Museum of Natural History,* 13: 1–10.

WILLIAMS, J. D., M. L. WARREN, JR., K. S. CUMMINGS, J. L. HARRIS & R. J. NEVES, 1993, Conservation status of freshwater mussels of the United States and Canada. *Fisheries,* 18(9): 6–22.

ZALE, A. V. & R. J. NEVES, 1982, Fish hosts of four species of lampsiline mussels (Mollusca: Unionidae) in Big Moccasin Creek, Virginia. *Canadian Journal of Zoology,* 60: 2535–2542.

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APPENDIX 1. Estimated sizes of restriction fragments used in the key (excluding fragments shorter than 100 bp).

Couplet	Pattern	Fragment Size (bp)	Couplet	Pattern	Fragment Size (bp)
1	A =	(275 – 285) + 185	5	0 =	415
	B =	305 + 185		A =	250 + 180
	C =	275 + 140*	6	A =	575 + 280 + 205
	D =	305 + 140**		A′ =	575 + 290 + 195
	E =	465 + 140	7	0 =	615
	F =	(495 – 505) + 140		A =	510
	G =	960	8	0 =	575
	H =	895		A =	370 + 225
	*anomalous *P. sintoxia* =		9	0 =	565
		205 + 140		A =	340 + 225
	**anomalous *A. plicata* =		10	A =	385 + 270 + 195
		305 + 170		A′ =	400 + 275 + 195
2	A =	350 + 230	11	A =	585
	B =	240 + 225 + 185		B =	555
3	A =	240 + 165	12	0 =	(590 – 620)
	B =	200 + 165		A =	430 + 190
	C =	235 + 195*	13	A =	455
	*anomalous *L. siliquoidea* =			B =	520
	1 =	245 + 205 + 170	14	0 =	(600 – 610)
	2 =	275 + 245 + 200		A =	370 + 240
4	A =	390 + 270 + 170	15	A =	500 + (445 – 460) + 195
	A′ =	375 + 265 + 170			
	B =	310 + 265 + 170		A′ =	515 + 460 + 195

APPENDIX 2. Partial matrix of restriction fragment patterns, by species.

Species	[PCR Product] Restriction Enzyme										
	[ITS-1]								[ITS 1-2]		[12S]	
	AccI	AvaI	BamHI	BstEII	HinfI	MboII	MspI	Sau96I	MboII	MspI	HaeIII	RsaI
Subfamily Anodontinae												
Alasmidonta marginata					C (8)		H (9)	D (2)		I (1)		
Anodontoides ferussacianus			O (3)		A (3)	O (3)	F (3)	C (3)	G′ (1)			
Lasmigona complanata			O (1)		B (1)	O (1)	F (1)	C (1)		G″ (1)		
Lasmigona compressa			O (4)		B (4)	A (4)	F (4)	C (4)		G″ (1)		
Lasmigona costata		O (1)	A (51)		B (1)	B (2)	F (51)	C (7)		G (1)		
Pyganodon grandis		O (1)	O (5)		B (5)	O (5)	F (5)	C (2)		G (5)		
Strophitus undulatus			O (1)		D (18)	B (1)	G (22)			H (1)		
Subfamily Ambleminae												
Tribe Amblemini												
Amblema plicata		A (1)	O (1)		O (1)		D(39)/B(1)	A (1)		D′ (1)		
Quadrula cylindrica			O (1)		O (1)	O (1)	E (9)	B (9)		F (1)		
Tribe Pleurobemini												
Elliptio dilatata	A (4)	A (1)	O (1)	A (13)	O (1)	O (1)	C (13)	B (13)		E (1)		
Fusconaia subrotunda	A (3)			O (6)		O (1)	C (6)	B (3)		E′ (6)		
Pleurobema clava	A (1)	A (1)		O (3)		O (1)	C (3)	B (1)		E (3)	A (1)	
Pleurobema sintoxia	A (4)	A (1)		O (5)		O (2)	C(4)/I(1)	B(4)/E(1)		E (5)	A (1)	
Tribe Lampsilini												
Actinonaias ligamentina	A (2)	A (10)	O (2)	O (1)	O (1)	O (1)	A (18)	A (18)	A (1)	A (1)	B (18)	
Epioblasma torulosa	A′? (2)	A (2)	O (3)	O (1)	O (1)	O (1)	A (4)	A (4)	A(2)/A′(2)	A (4)	A (4)	O (4)
Epioblasma triquetra	A (2)	A (2)	O (3)	O (2)	O (1)		A (3)	A (3)	A′ (3)	A (3)	A (3)	O (3)
Lampsilis cardium	A (1)	A (8)	O (2)	O (2)	O (2)	O (4)	A (8)	A (8)	A (2)	A′ (6)	A (8)	O(5)/A(1)
Lampsilis fasciola		A (2)	O (2)	O (1)	O (2)	O (3)	A (5)	A (5)	A (1)	B (2)	A (5)	A (5)
Lampsilis ovata	A (1)	A (7)	O (2)	O (1)	O (2)	O (4)	A (7)	A (7)	A (2)	A′ (7)	A (7)	O(6)/A(1)
Lampsilis siliquoidea	A (1)	A (2)	O (2)	O (2)	O (2)	O (3)	A (30)	A (30)	A (1)	A′ (1)	C(28)/D(2)	
Ligumia nasuta					O (2)		B (11)	A (2)		D (1)		
Ligumia recta		O (4)	O (1)		O (2)	O (4)	A (4)	A (4)		A′ (4)	A (4)	O(3)/B(1)
Ptychobranchus fasciolaris	A (13)					O (2)	A (13)	B (13)		C (1)		
Villosa fabilis	O (17)	A (1)	O (1)				A (17)	B (17)		A (1)		
Villosa iris	A (2)	A (2)		O (1)	O (2)	O (4)	A (6)	A (6)	A (1)	A (6)	A (6)	A (6)

A, B, . . . = restriction fragment patterns; O = no restriction sites present; (#) = sample size
Note: Many results presented in Appendix 2 are unreplicated.