Molecular Ecology (1994) 3, 183–185

TECHNICAL NOTE

Identification of freshwater mussel glochidia on host fishes using restriction fragment length polymorphisms

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North America's freshwater mussel (Bivalvia: Unionoidea) fauna is declining precipitously in richness and abundance. Two hundred and thirteen of the 297 recognized species and subspecies are endangered, threatened, or of special concern (Williams *et al.* 1993). *In-situ* preservation and laboratory propagation are hampered by severe inadequacies in knowledge of unionid reproductive requirements. The glochidia larvae of virtually all North American unionids are obligate parasites of fishes (Tucker 1927). Host species have been suggested for only 25% of the North American fauna, and many proposed relationships are questionable (Hoggarth 1992). To date, host lists have been derived primarily via artificial inoculation of putative hosts (e.g. Zale and Neves 1982) or morphological identification of glochidia on fishes (e.g. Wiles 1975). The former is time-consuming, ill-suited to systems with many potential host species, and insensitive to microhabitat, morphological, and behavioural factors that might modulate unionid-fish interactions *in situ*. The latter is problematical because glochidia are less than 1 mm in diameter; encystment makes observation difficult and may influence their shapes in unpredictable ways (Wiles 1975).

We are developing a new method for identifying glochidia attached to fishes, utilizing restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) products. A diagnostic suite of restriction sites is sought for each mussel species in the aquatic system of interest. Glochidia on host fishes are then identified based on the restriction sites they possess.

Our preliminary objective in developing this method was to identify a region of the genome that would (i) allow easy separation of unionid DNA and host fish DNA, and (ii) contain sufficient variation to permit species-level discrimination based on RFLPs. The former is essential because there is a threat of host contamination of PCR products obtained from glochidial DNA, given the extreme sensitivity of PCR and the highly conserved nature of the primers being employed.

Keywords: glochidial identification, ITS–1, RFLP analysis, Unionoidea

Received 21 July 1993; revision received 8 October 1993; accepted 13 October 1993

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The aquatic system for which we are developing our method is French Creek, a fourth-order tributary to the upper Allegheny River in north-western Pennsylvania. The French Creek drainage harbours 25 unionid species and at least 60 fish species. Two of its unionids, *Epioblasma torulosa rangiana* (I. Lea) and *Pleurobema clava* (Lamarck), are endangered and have no known hosts.

Fishes and adult unionids were collected throughout French Creek by seining and snorkelling, respectively. Fishes were transported to the laboratory alive and were maintained in aquaria for at least 1 week before they were killed and frozen at − 80 °C; any unencysted glochidia were presumed to have been sloughed off by fishes during the holding period. Live unionids were transported to the laboratory on ice, where they were either killed and frozen at − 80 °C or maintained in aquaria. Tissue samples were cut from the foot of frozen specimens. Glochidia of known identity were obtained from marsupia of frozen gravid females. Tissue samples were obtained from live specimens by relaxing their adductor muscles in soda water and clipping off small (5–50-mg) pieces of foot; this procedure did not appear to have a negative effect on survival.

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. Attached glochidia were excised over ice and homogenized in 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, then diluted to a final volume of 50 μL with sterile distilled water (after Martin *et al.* 1992).

The first internal transcribed spacer (ITS-1) region of the nuclear ribosomal DNA was amplified using PCR. We concentrated on ITS-1 for three reasons:

1 its length varies considerably across taxa, minimally from 343 bp (Kupriyanova & Timofeeva 1988) to 1095 bp (Gonzalez *et al.* 1990);

2 the highly conserved 18S and 5.8S flanking regions can be exploited for designing primers;

3 the rate of ITS-1 sequence evolution is intermediate (e.g. Mindell & Honeycutt 1990), hence species-level differences in restriction sites are likely.

The primers 5′TAACAAGGTTTCCGTAGGTG-3′ (18S region) and 5′AGCTRGCTGCGTTCTTCATCGA-3′ (5.8S region) were used with 1 μL of template DNA (diluted 0–1000×) and 2.5 units of Perkin–Elmer–Cetus *Taq* polymerase under manufacturer-recommended conditions, in 50- or 100-μL reactions. For attached glochidia, 3–10 μL of template were used. Thirty-four amplification cycles were performed (1 min at 93 °C, 1 min at 50 °C, 2 min at 72 °C) followed by one cycle with increased extension time (9 min). To date, amplification has been successful in 13 of 17 glochidia.

Restriction enzyme digests were performed in 10-μL reaction volumes consisting of 1 μL of PCR product, 5–10 units of restriction enzyme, and 1 μL of the buffer supplied by the manufacturer. Digests were carried out at 37 °C for 4–18 h. Restriction fragments and uncut PCR products were assayed on a 2.0% agarose gel stained with ethidium bromide.

Without exception, the ITS-1 regions of the fishes we examined are markedly different in length from the unionids'. Examination of single individuals yielded a product of approximately 690–710 bp for all darters examined but *E. maculatum*, for which it is approximately 410 bp (Fig. 1). Among the unionids, ITS-1 length is approximately 580–625 bp. Contamination of glochidial DNA by host fish DNA is thus easily ascertained. Furthermore, when darter ITS-1 PCR products are digested with *Msp*I, they yield restriction fragment patterns different from all unionid patterns (unpublished. data). Hence, even if the glochidial identification method we describe were applied to fishes whose ITS-1 regions are close to unionids in length (e.g. salmonids; Pleyte *et al.* 1992), detecting contamination should be possible by digesting the host's ITS-1 product alongside the products of the glochidia it harboured. Primer dimers and minor amplification products likewise do not interfere with glochidial identification.

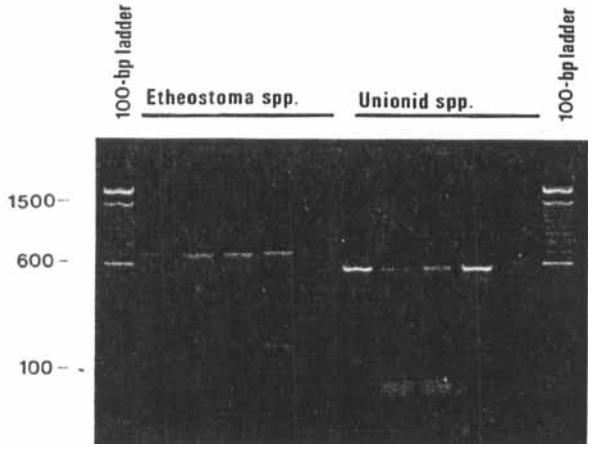

Fig. 1 Darter (*Etheostoma* species) and unionid ITS–1 amplification products. *Etheostoma* species are, from left, *E. blennioides*, *E. variatum*, *E. zonale*, *E. flabellare* and *E. maculatum*; unionid species are *Lampsilis siliquoidea* (Barnes), *Actinonaias ligamentina* (Lamarck), *Epioblasma torulosa rangiana*, *Amblema plicata* and *Lasmigona costata*.

The suitability of the ITS-1 region for distinguishing among unionid species is apparently high. Digestion of ITS-1 PCR products from single individuals of 12 unionid species with *Msp*I yields five different restriction fragment patterns (Fig. 2). Based on these patterns, 11 of 12 species can be identified to tribe. The sole exception is the lampsiline *Ptychobranchus fasciolaris* (Rafinesque), which exhibits a pattern indistinguishable from that of *Elliptio dilatata* (Rafinesque), in the tribe Pleurobemini. This corroborates Davis & Fuller's (1981) finding that *Ptychobranchus* is the lampsiline genus most similar to the Pleurobemini, based on immunoelectrophoretic studies. *Amblema plicata* (Say) and *Quadrula cylindrica* (Say) can be identified to species based on their *Msp*I restriction fragment patterns alone. Two additional restriction enzymes separate four more unionids. *Bam*HI cuts *Lasmigona costata* (Rafinesque) but not *Strophitus undulatus* (Say); *Sau*96I separates *Ligumia recta* (Lamarck) and *Villosa fabalis* (I. Lea) from each other and from the other lampsilines examined. *Acc*I, *Ava*I, *Bgl*I, *Bst*EII, *Hae*III, *Hin*fI, *Mbo*I, *Not*I, and *Pst*I add no further resolution. We are optimistic that sequencing the region in species we cannot yet differentiate will reveal additional restriction-site differences, and that the remaining 13 French Creek unionids will also prove distinguishable.

The ITS-1 PCR product of glochidia obtained from a gravid female *L. costata* exhibited the same pattern as that of the adult when digested with *Msp*I (Fig. 2). ITS-1 products from four individuals of *L. costata* collected at three different sites generated identical restriction fragment patterns when digested with *Msp*I, *Bam*HI, and *Sau*96I (unpublished data), suggesting low intraspecific variation in the ITS-1 region. We are currently examining more individuals and including specimens from elsewhere in the Ohio basin, to ensure that the diagnostic suites of restriction sites we identify are truly species-specific.

The molecular method for identifying glochidia described herein has enormous potential for yielding much-needed information about unionids patterns of host-fish use. It circumvents the aforementioned problems associated with traditional approaches to host-fish identification and does not entail sacrificing adult unionids. When developed fully, it promises to be relatively fast, reliable, and inexpensive. It can be used to address both the pressing

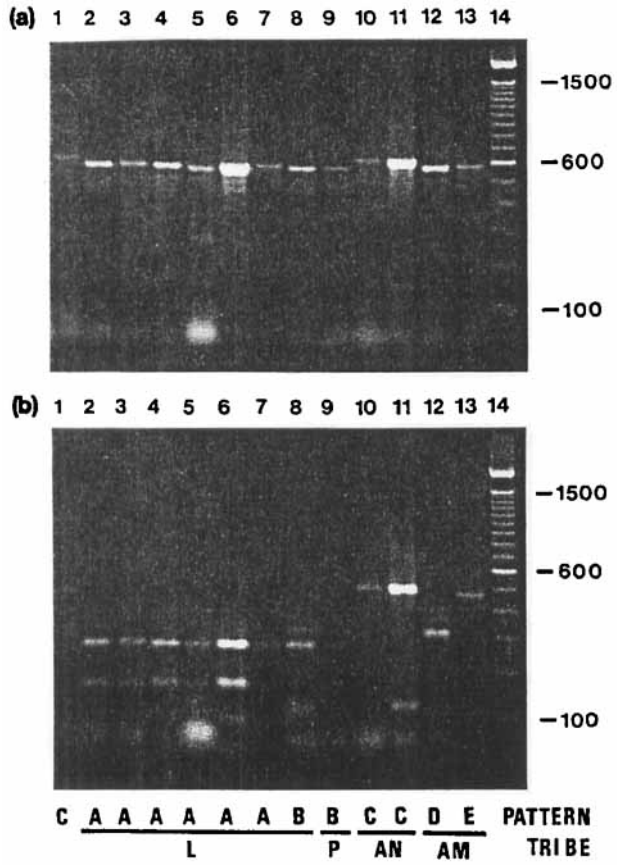

Fig. 2 (a) Uncut ITS–1 amplification products from 12 unionid species. (B) Restriction fragment length polymorphisms revealed by digestion of ITS–1 with *Msp*I. Pattern (A–E) separates most species into their respective tribes (see text). 1, glochidia obtained from marsupium of *Lasmigona costata*; 2, *Epioblasma triquetra* (Rafinesque); 3, *Epioblasma torulosa rangiana*; 4, *Lampsilis siliquoidea*; 5, *Actinonaias ligamentina*; 6, *Villosa fabalis*; 7, *Ligumia recta*; 8, *Ptychobranchus fasciolaris*; 9, *Elliptio dilatata*; 10, *Lasmigona costata*; 11, *Strophitus undulatus*; 12, *Amblema plicata*; 13, *Quadrula cylindrica*; 14, 100-bp ladder. Tribe AM = Amblemini, AN = (subfamily) Anodontinae, L = Lampsilini, P = Pleurobemini. Tribe designations follow Vaught (1989).

conservation challenge of establishing host-fish identities and issues of theoretical interest, such as patterns of host-fish partitioning among unionids. Moreover, the genetic data generated are potentially valuable to unionid systematists. Finally, the method is applicable to systems with large numbers of unionid and fish species.

Acknowledgements

Supported by a grant from the Pennsylvania Wild Resource Conservation Fund to J. R. Stauffer, Jr, and a National Science Foundation Graduate Research Fellowship to L. R. White. W. S. Sheppard provided primers. H.-Y. Han and C. L. Crego gave valuable advice and assistance in the laboratory. A. E. Bogan, M. J. Gutowski, and R. Shema helped collect specimens. The manuscript was critically reviewed by A. E. Bogan, M. J. Gutowski, and two anonymous reviewers.

References

Davis GM, Fuller SLH (1981) Genetic relationships among recent Unionacea (Bivalvia) of North America. *Malacologia*, **20**, 217–253.

Gonzalez IL, Sylvester JE, Smith TF *et al.* (1990) Ribosomal RNA gene sequences and hominoid phylogeny. *Molecular Biology and Evolution*, **7**, 203–219.

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

Kocher TD, Thomas WK, Meyer A *et al.* (1989) Dynamics of mitochondrial evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA*, **86**, 6196–6200.

Kupriyanova NS, Timofeeva MY (1988) Primary structure of the internal transcribable spacer of ribosomal genes of the loach *Misgurnus fossilis* L. Similarity in the organization of the major processing site in eukaryotes. *Molecular Biology (Kiev)*, **21**, 1000–1009.

Martin AP, Humphreys R, Palumbi SR (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 Science*, **49**, 2386–2391.

Mindell DP, Honeycutt RL (1990) Ribosomal RNA in vertebrates: evolution and phylogenetic applications. *Annual Review of Ecology and Systematics*, **21**, 541–566.

Pleyte KA, Duncan SD, Phillips RB (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.

Tucker ME (1927) Morphology of the glochidium and juvenile of the mussel *Anodonta imbecilis*. *Transactions of the American Microscopical Society*, **46**, 286–293.

Vaught KC (1989) *A Classification of the Living Mollusca*. American Malacologists, Inc., Melbourne, Florida.

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

Williams JD, Warren ML, Cummings KS *et al.* (1993) Conservation status of freshwater mussels of the United States and Canada. *Fisheries*, **18**, 6–22.

Zale AV, Neves RJ (1982) Fish hosts of four species of lampsiline mussels (Mollusca: Unionidae) in Big Moccasin Creek, Virginia. *Canadian Journal of Zoology*, **60**, 2535–2542.