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Source: Copeia, 2010(4):554-557. 2010.
Published By: The American Society of Ichthyologists and Herpetologists
DOI: 10.1643/CG-09-191
URL: http://www.bioone.org/doi/full/10.1643/CG-09-191

Reproductive Ecology of Nest Associates: Use of RFLPs to Identify Cyprinid Eggs

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In this study, we use restriction fragment length polymorphisms (RFLP) of the mtDNA ND2 gene to identify to species cyprinid eggs collected from a spawning aggregation substrate in the Pascagoula River system. A library was generated with inter- and intra-specific RFLP patterns for nine cyprinids: *Notropis baileyi, N. texanus, N. longirostris, N. amplamala, Nocomis leptocephalus, Luxilus chrysocephalus, Lythrurus roseipinnis,* and *Cyprinella venusta*. Eggs of three species were identified from a single nest, while only one species was observed spawning at the site. These results confirm that RFLP is an effective method for identifying unknown eggs of nest associates, and North American cyprinids in general.

NEST association, the reproductive strategy of spawning in the prepared substrate of another species' spawning site, is a visually stunning, yet poorly understood minnow spawning behavior. Of the North American cyprinids for which spawning behavior is known, 30% engage in nest association, either as a host or associate (Johnston and Page, 1992; Johnston, 1994a), and many exhibit vibrant nuptial coloration. Eggs and larvae of nest associates benefit from the parental care offered by the host as well as the physical structure of the nest site (Johnston, 1994a, 1994b). Host eggs may benefit from a dumping effect (Johnston, 1994b) or may be eaten by some nest associates (Fletcher, 1993); therefore, nest association is a symbiotic relationship with both mutualistic and parasitic manifestations.

Despite the studies mentioned above and the attention nest association has received over the past 60 years, some overarching questions remain: Have all participant nest associates within a particular community been identified? Do all species seen in aggregations over a particular nest site deposit eggs at that site? Much of what is known about the participants of nest association has been gleaned from diurnal observations of spawning aggregations, with as many as six species engaged in spawning behavior over a single nest site (Johnston and Page, 1992; Cashner, pers. obs.). Although participants in these aggregations exhibit spawning coloration and position jockeying, actual participation in spawning is unknown for most. Cooper (1980) reared larvae from eggs obtained from active *Nocomis micropogon* nests and verified up to five species from one nest. No other study has reported either the number, or the identity of species that have successfully spawned at a given aggregation site. This is partially because there are few distinguishing characters that allow for identification of cyprinid eggs, especially among species with similar breeding ecologies (Coburn, 1986; Platania and Altenbach, 1998; Ross, 2001; Boschung and Mayden, 2004). Thus, a reliable, affordable, and less time consuming (than rearing) egg identification protocol is needed.

Because mitochondrial DNA (mtDNA) is abundant in individual cells (thus eggs) and has a high degree of interspecific variation, it has been the primary source for genetics-based identification of fishes (Ward and Grewe, 1994; Pegg et al., 2006). Published molecular methods used to identify fish eggs are: direct sequencing (Pegg et al., 2006); multiplex-PCR (Hyde et al., 2005; Perez et al., 2005); PCR-SSCP (single strand conformation polymorphism; Garcia-Vazquez et al., 2006); and PCR-RFLP (restriction fragment length polymorphism; Chow et al., 1993; Cordes et al., 2001; Lin et al., 2002). Marine fishes have been the primary focus of these studies, and all methods, except PCR-RFLP and direct sequencing, have only been successful identifying a limited number of species.

Compared to direct sequencing, RFLP technique is inexpensive, and requires only basic technical skills to execute. With the appropriate mtDNA gene, it can also be used across a wide range of species. We have chosen to use the ND2 gene because communities of nest associates can be composed of closely related species. Therefore, we used a marker that was variable enough to detect interspecific variation using one set of endonucleases. Chow et al. (1993) were unable to distinguish between closely related species of lutjanids using the cytochrome *b* gene; thus, we felt that the higher mutation rate of ND2 could resolve species differences among North American cyprinids likely to be encountered during this study.

The purpose of this paper is to demonstrate that RFLPs can be used to determine species identity of cyprinid eggs within a rich community of nest associates. Identification of eggs using this technique provides researchers with a powerful tool to address a broad range of reproductive ecology questions. The objectives of this study are to: develop a RFLP library for a species-rich cyprinid community using restriction endonucleases that result in low intra-specific and high inter-specific variation in cut patterns; determine whether eggs can be identified reliably using this technique; and assess the applicability of this technique to investigations of spawning ecology of nest associates.

MATERIALS AND METHODS

RFLP library generation.—Known nest associates within the Pascagoula River drainage in Mississippi, USA, are the mound-building host, *Nocomis leptocephalus*, and the associate spawners, *Notropis baileyi* and *Luxilus chrysocephalus*. Six other common cyprinids are often collected with the aforementioned species: *Notropis texanus*, *Notropis longirostris*, *Lythrurus roseipinnis*, *Cyprinella venusta*, *Hybopsis winchelli*, and *Notropis amplamala*. Egg deposition and definitive spawning localities are not known for four of these species (*N. texanus*, *L. roseipinnis*, *H. winchelli*, and *N. amplamala*; Ross, 2001).

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Submitted: 16 October 2009. Accepted: 3 May 2010. Associate Editor: W. L. Smith.
 DOI: 10.1643/CG-09-191

Three to 20 individuals of each species were collected and preserved in 95% ethanol, with a total of 96 specimens assayed for RFLP patterns. Some specimens of *Notropis baileyi* and *Nocomis leptocephalus* were collected outside the Pascagoula drainage. Adult (known) DNA was extracted from fin tissue using a modified Chelex 100 extraction protocol using 50–100 ng of Proteinase K and approximately 25 ng of fin tissue (Walsh et al., 1991).

Amplification of the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene and surrounding regions was carried out by polymerase chain reaction (PCR) using primers situated in the glutamine and asparagine tRNAs (GLN and ASN from Kocher et al., 1995). Reactions were conducted in a total volume of 25 µl containing 0.5 µl 10 mM dNTPs, 1 µl 10 pM of each primer, 0.25 µl Taq DNA Polymerase, 0.75 µl 25 mM magnesium solution, and 2.5 µl 10× Taq buffer with magnesium. Reactions were carried out in an Eppendorf Mastercycler with a step-down cycle protocol adapted from Kocher et al. (1995): 94°C for 30 s; five cycles of 94°C for 30 s, 57°C for 30 s. and 72°C for 1 minute 15 s; five cycles with an annealing temperature of 56°C; five cycles with an annealing temperature of 55°C; and 20 cycles with an annealing temperature of 54°C; followed by a single 72°C final extension step for 10 min.

Endonuclease selection was based on 1047 bp of the ND2 gene for four species: *Notropis baileyi* (n = 3), *N. texanus* (n = 1), *Nocomis leptocephalus* (n = 2), and *Luxilus chrysocephalus* (n = 2; GenBank accession numbers EF613557, EF613558, EF613561, EF613563, EF613564, EF613566, EF613567, EF613581) that were direct sequenced with ABI BigDye reactions using the sequencing primers ND2B-L and ND2E-H (Broughton and Gold, 2000) and ASN (Kocher et al., 1995). Sequences were analysed via the Sequencher v4.5 (Gene Codes, Ann Arbor, MI) cut map feature to identify endonucleases that would result in high inter-specific and low intra-specific pattern variation. Endonucleases *Hinf*I and *Hha*I best fit the requirements and were compatible for double digestion protocol (New England Biolabs, Inc., Ipswich, MA).

Double restriction digestions were carried out in a 25 µl reaction containing 10–19 µl PCR product, one unit of each endonuclease, 2.5 µl 10× NEB 2 buffer, 0.25 µl of 10 mg/ml BSA, and autoclaved distilled water. Digestion occurred in an Eppendorf Mastercycler at 37°C for 3 h followed by a 20 min 80°C stop temperature. The total product was electrophoresed through a 3% Nu-Seive/Agarose gel at 83 volts for 2 h. Gels were stained with ethidium bromide, visualized on a UV transilluminator, and digitally photographed. To enhance scoring consistency, all samples were run adjacent to a NEB 100 bp ladder (New England Biolabs, Inc., Ipswich, MA). Cut patterns were visually assessed for all nine focal species. Bands smaller than 100 bp were not counted due to increased potential of inaccurate mobility.

In order to verify that RFLP banding patterns accurately represented ND2 gene nucleotide sequences, unique patterns within and among species were identified and representative individuals were direct sequenced for the ND2 gene (GenBank accession numbers EF613559, EF613560, EF613562, EF613563, EF613565, EF613568–83). The resulting sequences were subjected to NEBcutter (Vincze et al., 2003) to directly compare the predicted RFLP banding patterns with those observed.

Egg collection, DNA extraction, and RFLP procedure.—One spawning site was identified in Martin Branch, Pascagoula River drainage on 5 May 2006. In 15 minutes of observation time, only *N. baileyi* was observed engaged in spawning behavior over a gravel nest. Eggs were collected by positioning an aquarium net just downstream of the nest while the gravel was manually agitated to release the eggs buried within it. Approximately 500 eggs were collected from the nest and placed in 95% ethanol. Samples were placed in fresh ethanol within one day, and transferred to fresh ethanol twice more within the first week of collection.

Egg DNA extraction followed a more complicated protocol: first soaked in a container of autoclaved distilled water for approximately 20 minutes in order to re-hydrate and remove ethanol; then each egg was placed in a solution of 50 µl of egg extraction buffer (DeWoody et al., 2000: 10 mM Tris 8.0, 1mM EDTA pH 8.0, 25 mM NaCl) and 0.3 mg/ml Proteinase K, ground with a sterile toothpick, incubated in a 55°C water bath for 30 min then in a 95°C heat block for 2 min; the extraction solution was spun down in a microcentrifuge for 3–5 s, and 3 µl of the supernatant was used for PCR amplification.

Restriction digestions occurred in the same manner as described above, except that all digestions included 19 µl of PCR product. Eggs were identified to species by visual comparison with the previously obtained RFLP library. To verify identification, a random subset of eggs was direct sequenced using the primers outlined above and edited using Sequencher 4.5. The resulting sequences were compared to known ND2 sequences for each focal species (GenBank accession numbers EF613584–93).

RESULTS

Amplification of the mtDNA ND2 gene generated a product that was approximately 1400 bp in length. This product includes the ND2 gene and flanking tRNA regions. Observed RFLP banding patterns differed slightly from predicted patterns generated by NEBcutter (Vincze et al., 2003), and this is most likely because the nucleotide sequences initially analyzed did not include the flanking tRNA nucleotide sequences. Restriction fragment length polymorphism banding patterns produced from double digests of *Hinf* I and *Hha*I resulted in species-specific band patterns for the nine focal taxa. Intra-specific variation in cut patterns were observed in some species; however, in most cases they were due to variation in mtDNA nucleotide sequences for specimens from different river drainages as is the case for *Nocomis leptocephalus* and *N. baileyi*. Within the Pascagoula drainage, one cut pattern was recovered for most species (Fig. 1). Exceptions were *N. longirostris* (six patterns), *L. chrysocephalus* (three patterns), and *C. venusta* (three patterns), however, for each species, one pattern was typically dominant. Variation in ND2 nucleotide sequences for a subset of individuals with unique banding patterns supported the variation observed in RFLP cut patterns (Table 1).

A total of 217 eggs from the nest site at Martin Branch were analyzed. *Notropis baileyi* eggs accounted for 33% (n = 72) of the sample, while *Luxilus chrysocephalus* eggs made up 51% (n = 110), and the host species, *Nocomis leptocephalus*, eggs represented 16% (n = 35) of the sample. Direct sequencing of ten randomly selected eggs (GenBank accession numbers EF613584–93) showed 100% support for species identification using the RFLP library.

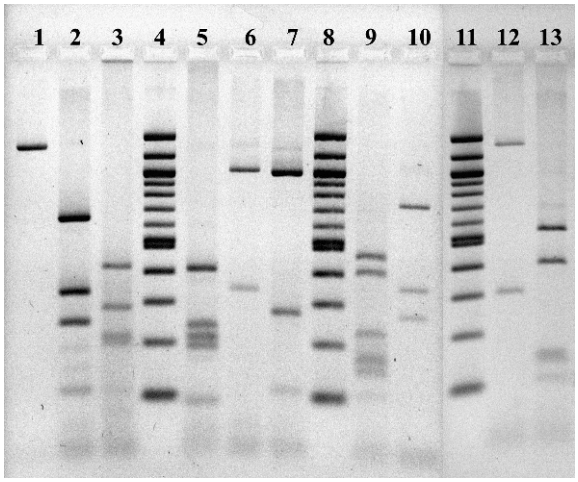

Fig. 1. RFLP banding patterns for all nine focal taxa. The most common patterns for species with variation are represented here. Lane 1 is a non-cut ND2 amplification used as a control marker; Lanes 4, 8, and 11 are NEB 100 bp ladders; Lane 2 is *Notropis baileyi*; Lane 3 is *Nocomis leptocephalus*; Lane 5 is *Notropis longirostris*; Lane 6 is *Notropis texanus*; Lane 7 is *Luxilus chrysocephalus*; Lane 9 is *Cyprinella venusta*; Lane 10 is *Lythrurus roseipinnis*; Lane 12 is *Hybopsis winchelli*; and Lane 13 is *Notropis amplamala*.

DISCUSSION

This study demonstrates that RFLP libraries of cyprinid breeding communities can be used to identify unknown eggs and assess participation in nest association. An RFLP library was generated for nine cyprinids, and all eggs successfully amplified produced RFLP patterns consistent with one of the nine species in the focal community. There were no ambiguous RFLP patterns either among known individuals or unknown eggs, illustrating that this technique is a reliable method for identification of cyprinid eggs, even within a diverse cyprinid community.

The methods described here rely on the amplification of the mitochondrial ND2 gene, a maternally inherited marker with a high mutation rate. Because the RFLP library is generated from known individuals within the focal community, incongruence between morphological identification and mtDNA can be attributed to hybridization. The frequency of occurrence of hybridization among specimens used in generating the RFLP library may require adjustments to the confidence researchers place in subsequent egg identification. No hybrids were detected in this study, therefore we are confident in the species identification of eggs examined.

In this study, only *Notropis baileyi* individuals were observed aggregating and displaying nuptial coloration over a *Nocomis leptocephalus* nest, while no *N. leptocephalus* individuals were observed at the site, nor were any collected during seining attempts. Restriction fragment length polymorphism analysis revealed that in addition to *N. baileyi*, *N. leptocephalus* and *Luxilus chrysocephalus* eggs were also present, confirming that this technique is useful for identifying members of a nest association even for species that have not been observed at a nest site. Surprisingly, the majority of eggs identified belonged to nest associates. With increased sampling (multiple nests within a community, and among communities), we may be able to determine whether this phenomenon is a result of *Nocomis leptocephalus* taking advantage of the selfish herd/dumping effect (Johnston, 1994b), *Notropis baileyi* and *L. chrysocephalus* using the nest site near the end of the hosts' spawning run, or competition among hosts and associates for spawning substrate.

Table 1. Observed and Predicted RFLP Patterns and Observed Sequence Variation for Those Species Which Displayed Intraspecific Variation.

Species	# Observed RFLP patterns	# Predicted patterns	Observed ND2 sequence variation (uncorrected p)
Nocomis leptocephalus	*3*	*3*	10.7–11.4%
Notropis baileyi	2	2	6%
Luxilus chrysocephalus	3	1	0–0.68%
Notropis longirostris	6	4	0–1.4%
Cyprinella venusta	*3*	*1*	0–0.1%

This technique is inexpensive and relatively easy to execute, and its application will make possible thorough characterization of participation in symbiotic spawning aggregations. Additionally, species-level egg identification will further investigations of inter-specific competition for egg deposition sites, timing of spawning by different members of the aggregation, and impacts of introduced nest associates on egg deposition of native species, thus providing a better understanding of the ecological and behavioral complexities of nest association in particular, and reproductive ecology of North American cyprinids in general.

MATERIAL EXAMINED

All specimens used in generating the RFLP library for this study are listed with TUMNH accession numbers and GenBank Accession numbers (where applicable) along with collection drainage below.

Cyprinella venusta (*n* = 10): TUMNH 198139, 199367 (GenBank EF613571), 199368–70, 199372 (GenBank EF613572–73), 199373–74, 200695, Pascagoula River, MS.
Hybopsis winchelli (*n* = 3): TUMNH 198138, 199397 (GenBank EF613582), 199398, Pascagoula River, MS.
Luxilus chrysocephalus (*n* = 10): TUMNH 198140, 199399 (GenBank EF613564), 199400, 199401 (GenBank EF613565), 199402–04, 200673, 200674–75 (GenBank EF613562–63), Pascagoula River, MS.
Lythrurus roseipinnis (*n* = 10): TUMNH 198142, 199419–21, 199422 (GenBank EF613574), 199423–27, Pascagoula River, MS.
Nocomis leptocephalus (*n* = 10): TUMNH 199665 (GenBank EF613566), 199519 (GenBank EF613569), Savannah River, GA; 20684 (GenBank EF613570), Alabama River, AL; TUMNH 198153, 199470 (GenBank EF613568), 200676, 200677 (GenBank EF613576), 200678–80, Pascagoula River, MS.
Notropis amplamala (*n* = 10): TUMNH 198143, 199375–77, 199378 (GenBank EF613583), 199379–84, Pascagoula River, MS.
Notropis baileyi (*n* = 10): TUMNH 196361, 196362 (GenBank EF613559), 196719 (GenBank EF613557), 196720, Tennessee River, AL; TUMNH 199716, 199732 (GenBank EF613560), 199744, 200670–72, Pascagoula River, MS.

Notropis longirostris (*n* = 20): TUMNH 198141, 199436–38 (GenBank EF613575–77), 199439, 199440–41 (GenBank EF61378–79), 199442–46, 199447 (GenBank EF613580), 199448–53, Pascagoula River, MS.
Notropis texanus (*n* = 10): TUMNH 198137, 199458–65, 199466 (GenBank EF613581), Pascagoula River, MS.

ACKNOWLEDGMENTS

We are grateful to the ASIH Raney Fund Award, the Graduate Women in Science Eloise Gerry Fellowship, the American Museum of Natural History Theodore Roosevelt Memorial Fund, and Sigma Xi Grants in Aid for funding support for this research. Additionally, we extend our appreciation to K. Piller and D. Hurley for laboratory space and resources necessary to perform this study. S. Woltmann and R. Blanton provided field assistance and comments on the development of this manuscript. Fishes and eggs were collected under unnumbered permits issued by the Mississippi Department of Wildlife, Fisheries, and Parks (2004–2006), permit numbers 3112 and 2042 issued by the Alabama Department of Conservation and Natural Resources (2004, 2005), and permit numbers 29-WMB-04-06 and 29-WSF-05-87 issued by the Georgia Department of Natural Resources (2004, 2005). Additionally, specimens were collected and used following Tulane University IACUC Protocols 0274-UT and 0327-UT.

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