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Biological and oceanographic insights from larval labrid (Pisces: Labridae) identification using mtDNA sequences

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Abstract. Polymerase chain reaction and direct comparison of mitochondrial DNA sequences from a cytochrome b gene fragment were used to identify two morphologically distinct larval types of *Xyrichtys*, a genus of tropical wrasse (Pisces: Labridae). Both larval types were collected during ichthyoplankton surveys on the Middle Atlantic Bight shelf in the summer of 1988. DNA sequence comparisons indicated that both types were larvae of *Xyrichtys novacula* (Linnaeus). Back-calculated birthdate distributions for those larvae collected on the Middle Atlantic Bight shelf demonstrated that the two larval types formed two distinct cohorts indicating a biological difference. The two distinct larval types may be a consequence of an ecophenotypic effect, or they may represent offspring from genetically distinct populations. These results emphasize that important biological and oceanographic information can be gained through the use of the polymerase chain reaction and DNA sequencing for larval identification.

Introduction

Many aquatic organisms have complex life histories in which the adult stage is preceded by morphologically distinct egg, larval and/or juvenile forms. Since Hjort (1914), biologists have studied the role of these early life history stages in the population dynamics of a variety of marine species (e.g. Walford 1938, Thorson 1950). Many marine ecologists have also examined pre-adult stages with regard to their importance in structuring intertidal and reef communities (Cowen 1985, Victor 1986a, Yoshioka 1986, Roughgarden et al. 1988). Eggs, larvae and juveniles are also used in systematic studies (Moser and Ahlstrom 1970, Moser et al. 1984, Fahay 1989) and in work examining the evolution of certain life history strategies (Morgan 1989, Strathmann et al. 1992). In addition to their biological utility, these early stages have been used as tracers of physical transport processes, providing qualities not available in other oceanographic tracers (Leis 1982, Mountain et al. 1989, Hare and Cowen 1991).

The study of early life history stages, however, is limited by the ability of scientists to make identifications to the species level. For marine fish species, even though a large amount of identification work has been done (e.g. Fahay 1983, Leis and Rennis 1983, Leis and Trnski 1989, Matarese et al. 1989), a high percentage of recognized species have larval stages that remain undescribed. Of the approximately 300 marine fish species reported from New Jersey (Able 1992), 29% have undescribed larval stages (M. Fahay personal communication). This problem is magnified in tropical faunas where, for example, of the more common Caribbean fishes (Randall 1983), 75% have larvae which remain undescribed (Richards 1990). It is obvious that to better utilize the early life history stages of fish, as well as invertebrates, efforts to identify and describe these stages must continue.

Recently, Hare and Cowen (1991) examined the transport processes responsible for the occurrence of larval *Xyrichtys* spp., a genus of tropical fish (Pisces: Labridae), in the Middle Atlantic Bight (MAB, Cape Hatteras, North Carolina to Cape Cod, Massachusetts). Specific identification could not be made, but larvae were tentatively assigned to *Xyrichtys novacula* based on species range information. *X. novacula* is found south of Cape Hatteras and into the Gulf of Mexico and Caribbean Sea, while the other two western Atlantic species, *X. martinicensis* and *X. splendens*, are found predominantly in the Caribbean and Gulf of Mexico, extending north only as far as southern Florida (Randall 1965).

Subsequent examination of these larvae, however, revealed the presence of two larval types which were distinguished based on the orientation of the ovoid eye (Fig. 1). There are several hypotheses regarding the occurrence of

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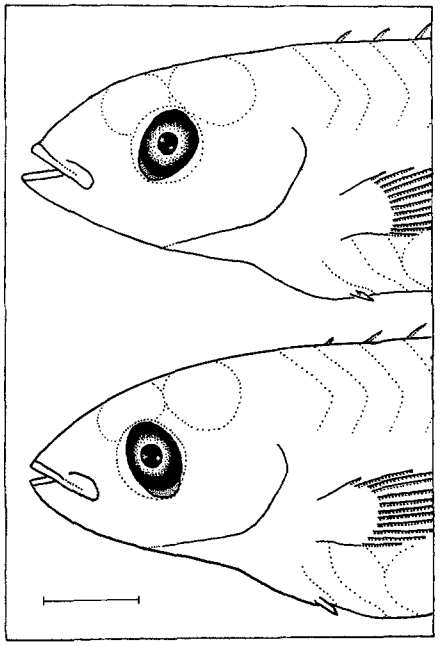

Fig. 1. *Xyrichtys* spp. Illustrations of the two larval eye types previously identified to the labrid genus *Xyrichtys* (Pisces: Labridae): ventral-forward eye type (top) and dorsal-forward eye type (bottom). Scale bar in lower left corner = 1 mm

two morphologically distinct larval types. One hypothesis is that these two types represent larvae of two *Xyrichtys* species. A second hypothesis is that these types belong to the same species but represent offspring from two distinct populations. Alternatively, these larvae may be ecophenotypes, with one eye type developing under one set of conditions and the other eye type developing under another set of conditions. A fourth hypothesis is that eye type is ontogenetically determined with the eye changing in orientation at some point during development. Lastly, it is possible that eye type is a random effect of collection and preservation.

Recent biochemical and molecular techniques provide promising approaches to identify the two types of *Xyrichtys* larvae to species. One example is direct comparison of DNA sequences facilitated by the synthesis of many copies of a particular fragment of DNA using the polymerase chain reaction (Kocher et al. 1989). This technique has recently been used to examine the phylogeny of cichlids (Meyer et al. 1990, Strumbauer and Meyer 1992) and neopterygian fishes (Normark et al. 1991), to determine the identification of adult *Thunnus* species (Bartlett and Davidson 1991), and to study the genetic structure of Atlantic cod (Carr and Marshall 1991), Atlantic salmon (McVeigh et al. 1991) and blue marlin (Finnerty and Block 1992). These studies suggest that it would be possible to identify the two larval types to species through a comparison of larval DNA sequences with sequences derived from all three western Atlantic *Xyrichtys* species. The cytochrome b gene is an appropriate choice for such inter-specific comparisons because it is evolutionarily conservative and previous studies have found sequences to be species specific (Meyer et al. 1990, Bartlett and Davidson 1991, Finnerty and Block 1992). Thus, our primary objective in the present study was to use direct DNA sequence comparisons to test the first hypothesis regarding the basis of these two larval eye types, namely, do the two morphologically distinct larval types represent two species of *Xyrichtys*. By using the polymerase chain reaction and DNA sequence comparisons to address this hypothesis, we also wanted to assess the general utility of these techniques for larval identification. A second objective was to examine the other four hypotheses regarding eye type through an analysis of temporal and spatial larval distributions in light of the DNA-derived species identifications.

Materials and methods

Larval collection and distributional analyses

Ichthyoplankton collections were made during four cruises aboard the NOAA Ship "Delaware II". Station locations and sampling procedures were given in Hare and Cowen (1991). Briefly, cruises were conducted approximately every other week from July 6 to August 12 1988 on the MAB shelf from Montauk Point, New York to Little Egg Inlet, New Jersey from the coast out to the 2000-m isobath. A total of 178 stations were sampled with an opening-closing, 505-μm mesh, 1 m^2 Tucker Trawl collecting at three discrete depths: 0 to 5, 5 to 10 and 10 to 15 m. Samples were split on board ship with half preserved in 95% ethanol for otolith analyses and half in 5% formalin for identification and measurement. Formalin samples were sorted and identified to the lowest possible taxonomic level.

Xyrichtys spp. larvae were measured with the aid of a computer enhanced video microscope image (Optical Pattern Recognition System, Biosonics, Seattle Washington) and orientation of the eye determined, either dorsal-forward or ventral-forward (see Fig. 1). From larval length, age was estimated using an age-length relationship,

$$\text{length} = 0.56 + (0.35 \times \text{age}) \qquad (r^2 = 0.85, p < 0.001)$$

determined previously using otolith ageing techniques (Hare and Cowen 1991). Approximately 30% of the individuals aged were dorsal-forward eye type, and the remainder were ventral-forward eye type. From these age estimates and the date of capture, birthdates were estimated for all measured larvae. In addition, frequencies of the two eye types were examined in terms of cruise of capture using an analysis of frequencies (Sokal and Rohlf 1981). To assess the possibility of ontogeny playing a role in determining eye type, an analysis of variance was used to examine the relationship between larval length and eye type (Sokal and Rohlf 1981).

Molecular techniques

Six individuals were used as tissue sources for polymerase chain reaction amplification and subsequent DNA sequencing. These included three larvae, one ventral-forward eye type (larva 1) and two dorsal-forward eye type (larvae 2 and 3), that were collected approximately 90 miles east of Cape May, New Jersey on August 8 1988. In addition, one adult specimen was used of each of the three western Atlantic *Xyrichtys* species. *X. martinicensis* (Cuvier and Valenciennes) was collected in Barbados, West Indies by R. K. Cowen and S. C. Sponaugle in 1991; *X. splendens* (Castelnau) was collected in Bermuda by R. K. Cowen and E. T. Schultz in 1992; *X. novacula* (Linnaeus) was borrowed from the Museum of Comparative Zoology (MCZ 90670) and was collected off the coast of Georgia, USA in 1971. All the material used for DNA sequencing in the present study was preserved in 95% ethanol except for the *X. novacula* specimen which was preserved in 70% ethanol.

DNA was extracted from ~5 mg of adult muscle tissue or a whole larva using standard phenol extraction procedures (Kocher et al.

1989), and samples were resuspended in 50 µl of TE (10 µ*M* Tris pH 8.0, 1 µ*M* Na_2EDTA pH 8.0). Primers for amplification were modified from the cytochrome b primers used by Kocher et al. (1989): 5′-GCCCCTCAGAATGATATTTGTCCTC-3′ and 5′-CCATCCAACATCTCAGCATGATG-3′. Polymerase chain reaction (PCR) protocol followed that of Kocher et al. (1989) except for the following differences. Two rounds of 25-cycle amplification were performed consisting of denaturation for 1 min at 93 °C, hybridization for 1 min at 50 °C and annealing for 2 min at 72 °C. The first round used 1 µl of raw DNA extract and the second used 1 µl of the end product of the first PCR reaction. Amplification resulted in a single band on an ethidium bromide stained 4% NuSieve agarose gel with an approximate length of 300 base pairs. Isolation of this band, blunt-ending of the fragment and cloning into M13 followed previously reported procedures (Zehr and McReynolds 1989). Both single-stranded and double-stranded DNA were sequenced from clones of the six samples using a commercial sequencing kit (Sequenase 2.0, United States Biochemical). Sequences have been deposited in GenBank and are under the following accession numbers: L16905 (*X. martinicensis*), L16906 (*X. novacula*), L16910 (*X. splendens*), L16907 (larva 1, ventral-forward eye type), L16908 (larva 2, dorsal-forward eye type) and L16909 (larva 3, dorsal-forward eye type).

Species identification

To make species identifications of the two larval types the cytochrome b sequences were used in a parsimony analysis. This analysis was done using the exhaustive search option of the Phylogenetic Analysis Using Parsimony software (PAUP 3.0, Swofford 1990). *Gomphosus varius*, a Pacific labrid, was used as an outgroup; cytochrome b sequence data for this species was obtained from Normark et al. (1991). Individual base pairs were used in the parsimony analysis because of the possibility of errors in the *Taq* polymerase amplification, reported to occur at a rate of 1 in 9000 (Saiki et al. 1988, Carr and Marshall 1991). Considering the cytochrome b fragment length (309 base pairs), the number of amplification cycles used (50) and the *Taq* error rate, 1 or 2 base pair errors were expected in our sequences. If an error were to occur at a second position in a codon, weighting the base positions in the parsimony analysis would magnify the effect of the error. Likewise, if the error resulted in a change in an amino acid, this would affect an analysis using amino acid sequences to a greater degree because of the lower number of characters (103 amino acids versus 309 base pairs). Thus, in this analysis each base pair was treated as an independent, equally weighted character.

Results

Analysis of larval collections

A total of 298 ventral-forward eye type larvae and 106 dorsal-forward eye type larvae were collected, enumerated and measured. Back-calculated birthdates demonstrated that the two larval eye types formed two distinct groups (Fig. 2). Ventral-forward eye type larvae, which were mostly collected during the first three cruises, formed an early-spawned cohort, whereas dorsal-forward eye type larvae, collected primarily during the fourth cruise, formed a distinct, later-spawned cohort (Fig. 2). Analysis of the number of the two eye types collected during each cruise found that frequencies were different between cruises (Table 1; $G_{adj} = 115.55$, $p < 0.001$). Simultaneous test procedures found that the first three cruises collected similar frequencies of the two eye types ($G_H = 0.26$, $p > 0.05$), but

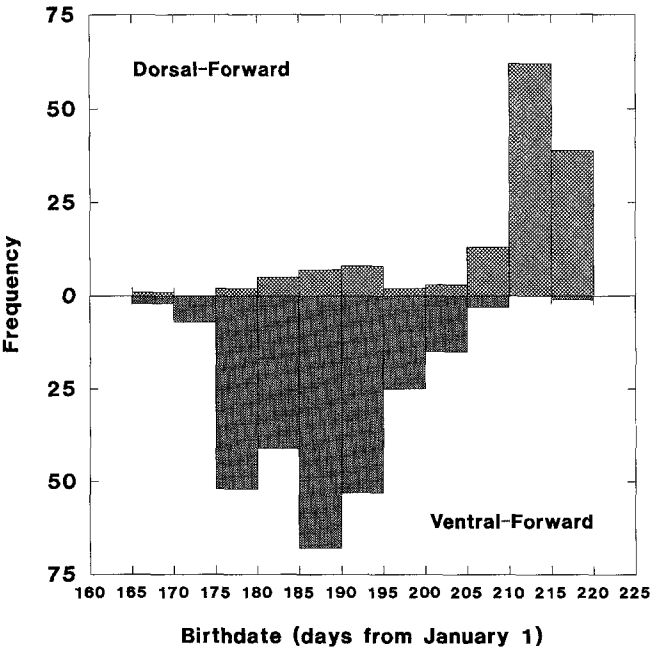

Fig. 2. *Xyrichtys* spp. Back-calculated birthdates of two morphologically distinct types of larval *Xyrichtys* spp. collected during four Middle Atlantic Bight cruises in the summer of 1988. Top panel shows birthdates for dorsal-forward eye type larvae and bottom panel shows birthdates for ventral-forward eye type larvae

Table 1. *Xyrichtys* spp. Number of ventral-forward and dorsal-forward eye type larvae collected during four ichthyplankton cruises on the Middle Atlantic Bight shelf in the summer of 1988

Cruise	Dates	Ventral-forward eye type	Dorsal-forward eye type
DEL-3-88	6–9 Jul	64	6
DEL-4-88	16–22 Jul	123	13
DEL-5-88	29 Jul – 2 Aug	59	7
DEL-6-88	8–12 Aug	52	80

the fourth cruise collected more of the dorsal-forward eye type larvae (minimum $G_H = 49.92$, $p < 0.05$, comparing fourth cruise to third cruise). A two-way ANOVA testing the effect of eye type and cohort on larval lengths found no significant effect of eye type ($F_S = 2.31$, $p > 0.1$) and no significant interaction effect between eye type and cohort ($F_S = 0.19$, $p > 0.1$). There was, however, a significant effect of cohort ($F_S = 12.13$, $p < 0.001$), primarily due to the fact that the first, early-spawned cohort was sampled during the first three cruises allowing for them to grow, whereas, the second, later-spawned cohort was sampled only when it first arrived on the MAB shelf. This analysis indicates that eye type is not affected by ontogeny (Fig. 3) and is predominantly a function of cohort (Fig. 2).

Species identification

DNA sequences of the 309 base pair portion of the cytochrome b gene were obtained for all specimens, except for *Xyrichtys splendens*, for which 226 base pairs were se-

quenced (Fig. 4). Sequences demonstrated that both larval types and *X. novacula* were genetically very similar with a maximum difference of four base pairs (Table 2). There were, however, substantial sequence differences between the three adult specimens, as well as between the larval types and *X. martinicensis* and *X. splendens*. Larvae were different from *X. splendens* at about 31% of base pairs sequenced and from *X. martinicensis* at about 30% of base pairs (Table 2). Of the base pair differences identified, the number of transitions (A-G or C-T) approximately equaled the number of transversions (A-C, A-T, G-C or G-T) except between *X. martinicensis* and *X. splendens* where transitions were 63% of the observed base differences (Table 3).

Cytochrome b sequence data indicated phenetically that both morphological types were larvae of *Xyrichtys novacula* and the parsimony analysis verified this finding (Fig. 5). The exhaustive search found the shortest trees to be 152 steps and the longest trees to be 245 steps. There were six trees which were 152 steps and nine trees which were 153 steps. These 15 trees differed only in the relationships between the larvae and *X. novacula*. Therefore, to establish the relationship between the larvae and the three western Atlantic species of *Xyrichtys*, a strict consensus of the six shortest trees was determined. This analysis demonstrated that *X. novacula* and the three larval types formed a monophyletic group with *X. martinicensis* and *X. splendens* forming the sister group (Fig. 5). With the data at hand, however, it is not possible to resolve the relationships between the larvae and *X. novacula*.

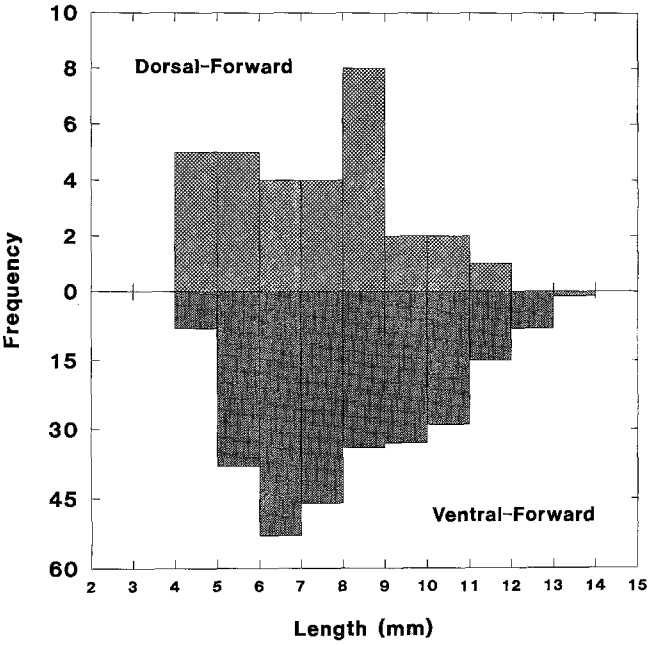

Fig. 3. *Xyrichtys* spp. Length frequency distribution of two morphologically distinct types of larval *Xyrichtys* spp. from the early-spawned cohort collected during four Middle Atlantic Bight cruises in the summer of 1988. Top panel shows lengths of the dorsal-forward eye type larvae and bottom panel shows lengths of ventral-forward eye type larvae

Table 2. *Xyrichtys matinicensis*, *X. novacula*, *X. splendens* and *Gomphosus varius*. Number of base pair differences (above diagonal) and percent base pair differences (below diagonal) in a 309 base pair cytochrome b fragment of the western Atlantic species of *Xyrichtys*: *X. martinicensis* (XM), *X. novacula* (XN) and *X. splendens* (XS); specimens of the two larval types of *Xyrichtys*: ventral-forward eye type (L1) and dorsal-forward eye type (L2 and L3); and of *Gomphosus varius* (GV), a Pacific labrid. Percent base pair differences based on number of comparable positions: 309 base pairs for *X. martinicensis*, *X. novacula*, and the three larval specimens, 285 base pairs for *G. varius* and 226 base pairs for *X. splendens*

Species	XM	XN	XS	L1	L2	L3	GV
X. martinicensis	–	92	38	92	92	91	53
X. novacula	29.77	–	70	4	3	1	80
X. splendens	16.81	30.97	–	69	70	69	39
Larva 1	29.77	1.78	30.53	–	3	3	79
Larva 2	29.77	1.33	30.97	0.97	–	2	80
Larva 3	29.45	0.44	30.53	0.97	0.65	–	79
G. varius	18.60	28.07	17.26	27.72	28.07	27.72	–

Table 3. *Xyrichtys martinicensis*, *X. novacula*, *X. splendens* and *Gomphosus varius*. Number of base substitutions that were transitions (above the diagonal, left-hand number) and transversions (above the diagonal, right-hand number) and the percent transitions of all base substitutions observed (below diagonal) in cytochrome b sequences of the western Atlantic species of *Xyrichtys*: *X. martinicensis* (XM), *X. novacula* (XN) and *X. splendens* (XS); and the Pacific labrid *G. varius* (GV)

Species	XM	XN	XS	GV
X. martinicensis	–	46/46	24/14	30/23
X. novacula	50.00	–	35/35	43/37
X. splendens	63.16	50.00	–	19/20
G. varius	56.60	53.75	48.72	–

Discussion

From mtDNA sequence comparisons, both larval eye types were identified as *Xyrichtys novacula*, thereby meeting our first objective. Cytochrome b sequences of larvae and all known western Atlantic *Xyrichtys* species demonstrated that the larvae and *X. novacula* formed a monophyletic group, allowing rejection of the hypotheses that larvae were *X. martinicensis* or *X. splendens*. Thus, unless any new species of *Xyrichtys* are found in the western Atlantic, we can conclude that both eye types are larvae of *X. novacula*. Given the amount of sequence divergence between species, we can also conclude that in general, DNA sequence comparisons of the cytochrome b fragment is a very useful technique for species-level larval identification.

Patterns of cytochrome b sequence divergence found in the genus *Xyrichtys* differ from those found in other groups of fish. McVeigh et al. (1991) found at 5% difference between the cytochrome b sequences of two species of *Salmo* (trout). Within the genus *Thunnus*, Bartlett and Davidson (1991) found about a 4% difference between species. The maximum sequence divergence between closely related genera of African cichlids studied by Meyer

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     1                                                                  60
XN   aac ttc ggc tct ctc ctt ggc gcc tgc ctg atc ctc caa atc acc aca gga cta ttc cta
L1   ... ... ... ..a ... .aa ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L2   ... ... ... ..a ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L3   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
XM   ..t ..t ... ... ... t.a ..t ctt ... t.c tct tct ... ..t ct. ... ..c ..c ... ..c
XS   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
GV   --- --- --- --- --- --- ..a ctt ... ..a gc. tc. ..g c.t ct. ..g ..c ..c ... ..g

    61                                                                  120
XN   gcc atg cac tac tca cca gac gcc tca acc gcc ttt tca tca atc gcc cac atc act cga
L1   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L2   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L3   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
XM   ..a ..a ... ... a.. t.. ... att g.t ..t ... ... ..c ..t g.a ... ... ... tgc ...
XS   --- --- --- --- --- --- --- --t g.c ... ..t ..c ..c ..c g.g ... ... ..t tgc ..g
GV   ... ... ... ... a.c t.. ... att g.t ..a ... ..c ... ..c g.t ... ... ..t tgc ...

   121                                                                  180
XN   gac gta aat tat ggc tga atc atc cgc tac ctt cac gcc aat ggc gcc tca ata ttc tct
L1   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .t.
L2   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .t.
L3   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .t.
XM   ... ..c g.c ..c ... ... c.g ... ..a a.. a.g ... ... ..c ..a ..a ..c t.c ... .tc
XS   ... ... ..c ..c ... ... c.g ... ..t a.. a.a ... ... ..c ... ..a ..t t.c ... .tc
GV   ... ..c ..c ... ... ... c.a ... ..t a.. a.a ..t ... ... ..a ..a ..t t.c ... .t.

   181                                                                  240
XN   atc tgc ctc ttc cta cac atc gga cga ggc cta tat tac gga tca ttt ctc tac tca gaa
L1   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L2   ... ... ... ... ... ... ... ..g ... ... ... ... ... ... ... ... ... ... ... ...
L3   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
XM   ... ... a.. .a. a.. ... ..t .cc ..a ..a ..t ..c ... ..g ..t .ac ... ... aa. ..g
XS   ... ..t a.t .a. a.g ... ..t .c. ... ..a ..g ..c ... ... ..c .ac ..a ... aa. ...
GV   ... ... a.. .a. ..g ... ... ... ... ... ..t ..c ... ... ... .ac ... ... aa. ...

   241                                                                  300
XN   acc tga aac atc ggc att atc ctc ctg ctt gca act ata gca aca gcc ttc ata ggc tat
L1   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L2   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
L3   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
XM   .gg ... ... ..t ..t g.a g.. ... ..c ..g ttg gt. ... at. ..* ... ... g.. ... ..c
XS   ... .c. ... ... ..a g.a g.c ..g ..c ..c ctt gt. ..g atg ..t ..t ..t c.. ... ..c
GV   ..a ... ... ... ... g.c g.t ..a t.a ... ct. gtc ..g at. ..t ... ... g.t ..a ..c

   301         309
XN   gtc ctc ccg
L1   ... ... ...
L2   ... ... ...
L3   ... ... ...
XM   ... ... ..a
XS   ... ..t ..a
GV   ... .-- ---
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Fig. 4. *Xyrichtys martinicensis*, *X. novacula*, *X. splendens* and *Gomphosus varius*. Mitochondrial DNA sequences of a 309 base pair fragment of the cytochrome b gene. Full sequence given for *X. novacula* (XN). For the other specimens: *X. martinicensis* (XM); *X. splendens* (XS); the three larval specimens (L1, L2, and L3), and *Gomphosus varius* (GV), dots mean sequence same as *X. novacula*, dashes mean no sequence data and asterisks mean base position not resolved through sequencing. Larva 1 was a ventral-forward eye type and larvae 2 and 3 were dorsal-forward eye type. *G. varius* sequence was obtained from GenBank (M64896) as reported by Normark et al. (1991)

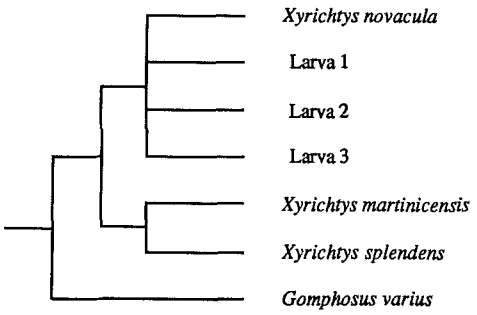

Fig. 5. *Xyrichtys martinicensis*, *X. novacula*, *X. splendens* and *Gomphosus varius*. Evolutionary tree for all western Atlantic species of *Xyrichtys* and the three larval specimens. Tree based on parsimony analysis of the cytochrome b sequences treating each base position as an independent, equally weighted character. Tree represents a strict consensus of the six lowest step trees. *G. varius* used as an outgroup and selected to be monophyletic with respect to the genus *Xyrichtys* in this analysis

et al. (1990) was 16%. Among billfish genera (*Istiophorus*, *Makaira*, *Tetrapturus* and *Xiphias*), Finnerty and Block (1992) reported a maximum difference of approximately 18%, with differences within the genus *Makaira* at approximately 4%. Thus, differences between *X. martinicensis* and *X. splendens* (~15%; Table 2) and between these two and *X. novacula* (~30%; Table 2) are greater than that found within other fish genera.

The ratios of transitions to transversions found in our study are also different than that found in other groups of fish. Within Atlantic cod (*Gadus morhua*) cytochrome b, Carr and Marshall (1991) found that 82% of the base substitutions were transitions. Finnerty and Block (1992) found 94% of base substitutions in the blue marlin (*Makaira nigricans*) to be transitions. Bartlett and Davidson (1991) found that 86% of the base substitutions within the genus *Thunnus* were transitions. Comparing two genera of cichlids (*Cichlasoma* and *Julidochromis*). Kocher et al. (1989) found on average 68% of base substitutions to be transitions. This pattern of transition bias is also found in other regions of fish mtDNA (Shedlock et al. 1992) and in other groups of animals (Brown et al. 1982. Kocher et al. 1989). Between species of *Xyrichtys*, however, we found little transition bias with values ranging from 50 to 63% (Table 3).

The large sequence divergences and low transition to transversion ratios found in the present study suggest that for the species analyzed, the cytochrome b fragment is saturated with substitutions and multiple events at a given site are possible. Sequence divergence is believed to increase rapidly following speciation, but then slows after approximately 15% overall divergence (Moritz et al. 1987). Likewise, transition to transversion ratios are initially high but decrease as sequences diverge (Moritz et al. 1987). The high sequence divergence and low transition bias found between species of *Xyrichtys* suggests that the genus itself is quite old, at least 15 million yr (see Fig. 1 in Moritz et al. 1987). Through fossil evidence it has been suggested that most Perciform groups, the order which contains the Labridae, had already formed by the Eocene, approximately 50 million yr ago (Carroll 1987, Patterson 1993). The first known labrid is from the acanthomorph fauna of Monte Bolca (Blot 1980), and the major labrid lineages were already differentiated in the Eocene (Choat and Bellwood 1991). Our data suggest that one modern labrid genus had evolved at least 15 million yr ago providing further evidence for the idea that the modern forms of labrids evolved rapidly and have changed little over recent geological time (Choat and Bellwood 1991).

In addition to considering the results of the present study molecular and evolutionary perspectices, the data presented here can also be used to address the immediate physical question of the mechanism by which larval *Xyrichtys novacula* are transported from South Atlantic Bight (SAB) spawning grounds to the MAB shelf. Back-calculated birthdates and frequency analysis of eye types collected over time clearly demonstrate that the two larval types formed temporally distinct cohorts. Comparison of these back-calculated birthdates with the larval distributions presented in Hare and Cowen (1991) indicates that the larval types were also spatially distinct. The early-spawned cohort was initially collected over the MAB slope in the vicinity of Hudson Canyon. During the following two cruises, larvae of the first cohort were found southwest and northeast of their original location along the shelf break, as well as across the shelf. By the last cruise most of the early-spawned cohort had crossed onto the shelf, while the second cohort appeared at the offshore edge of the area sampled, southwest of Hudson Canyon (see Fig. 2 in Hare and Cowen 1991).

Two distinct cohorts arriving at the MAB shelf edge at distinct times and locations provides evidence for two distinct transport events. Separation of these two events could have occurred at any step in the transport route described by Hare and Cowen (1991). *Xyrichtys novacula* larvae are incorporated into Gulf Stream associated flows from spawning locations on the SAB shelf, and after being transported northeastward via the Gulf Stream, they are transported across the Slope Sea to the MAB shelf edge via warm-core ring streamers. Evidence for two distinct events indicates that either two parcels of SAB shelf water were incorporated into Gulf Stream associated flows or that there were two distinct interactions between the Gulf Stream and the warm-core ring responsible for the transport of larvae across the slope. From synoptic satellite imagery it does not appear that there were two distinct streamer events during the period of cruises in the summer of 1988 (J. Churchill personal communication). Therefore, the separation of the two cohorts probably occurred at their incorporation into the Gulf Stream. Analysis of this hypothesis must wait for the distribution of the two larval eye types to be determined in the region of Cape Hatteras, but it is possible that these morphologically distinct larvae could be used as biological tracers of the mechanisms and frequency of entrainment of SAB shelf water into the Gulf Stream. Such an approach would assist in our understanding of larval transport in situations when Gulf Stream entrainment represents a loss of larvae from adult populations (e.g. *Bothus* spp., *Ioglossus calliurus*, *Syacium papillosum* and *Xyrichtys novacula*), and in situations when Gulf Stream entrainment is part of the life cycle (e.g. *Anguilla rostrata*, *Conger oceanicus* and *Pomatomus saltatrix*).

Using *Xyrichtys novacula* larvae as tracers, however, requires that the biological basis of larval eye orientation be understood. Comparison of cytochrome b sequences permits rejection of the hypothesis that the larvae represent two *Xyrichtys* species. Consideration of the spatial and temporal distributions allows two alternative hypotheses to be rejected, namely, eye type is not an artificial effect of preservation, and eye orientation does not change ontogenetically. Frequency analysis of eye types and back-calculated birthdates show a systematic pattern in the occurrence of the two larval eye types (Table 1, Fig. 2) which would not be predicted if eye type were a random effect. Examination of the length-eye orientation relationship demonstrates that eye type does not vary with length, indicating that this morphological difference is not ontogenetic (Fig. 3). Thus, two hypotheses remain at this time, either eye type is an individual character determined by some component of the environment or a population level character representing genetic differences. This dichotomy can be resolved through further DNA sequencing to test whether or not the two eye types differ genetically. The sequences of *X. novacula* exhibited some intra-specific variation (~1%), but sample sizes were small and the level of variation found was within the range expected due to PCR errors (0.3 to 0.6%). Thus, to address the issue of whether the two eye types are genetically distinct, it would be necessary to sequence DNA from a region which will show more intra-specific variation, for example the control region of the mtDNA genome (Meyer et al. 1990, Shedlock et al. 1992) or the first internal transcribed spacer of ribosomal DNA (Pleyte et al. 1992).

Eye shape is considered to be genetically determined as evidenced by its consistency within phylogenetic groups (Moser and Ahlstrom 1970, Weihs and Moser 1981). *Xyrichtys novacula* larvae, however, are in the plankton for 38 to 62 d (Victor 1986b) and have been collected 1 000 km northeast of their adult range (Hare and Cowen 1991). Thus, if orientation differences are genetically determined, the question is raised as to how population differences are maintained in the face of a huge dispersal potential? Perhaps intra-specific morphological and genetic differences can be used as population markers to determine actual larval dispersal from particular populations. This can be accomplished by combining molecular techniques with physical and biological oceanography. Such an approach would allow the biological and physical bases of evolution and maintenance of population discreetness to be examined in the context of the large potential gene flow created by planktonic early life history stages.

Thus, we feel that there are many benefits of using mtDNA sequences for larval identification. In the present study, species identifications of the two larval types were made and a phylogeny for the western Atlantic *Xyrichtys* species was proposed. It was also suggested that two distinct transport events were responsible for the disjunct distributions of the two morphologically distinct larval types, possibly allowing these eye types to be used in the future as tracers for the entrainment of SAB water into Gulf Stream associated flows. The biological factors responsible for the two larval types were also addressed resulting in the rejection of three of the five hypotheses initially proposed and further DNA sequencing would discriminate between the remaining two. We are not advocating the replacement of traditional larval identification techniques, but we believe that direct DNA sequence comparisons efficiently identify small numbers of larvae. In combination with insights from physical oceanographic data and temporal and spatial larval distributions, this technique can also provide information on population structure, the sources of dispersing larvae, and the physical mechanisms of dispersal.

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