Identification of larval sea basses (*Centropristis* spp.) using ribosomal DNA-specific molecular assays*

Mark W. Vandersea (contact author)[1]
R. Wayne Litaker[1]
Katrin E. Marancik[2]
Jonathan A. Hare[2]
Harvey J. Walsh[3]
Siya Lem[4]
Melissa A. West[5]
David M. Wyanski[6]
Elisabeth H. Laban[1]
Patricia A. Tester[1]

Email address for Mark W. Vandersea:
Mark.W.Vandersea@noaa.gov

[1] National Ocean Service, NOAA
Center for Coastal Fisheries and Habitat Research
101 Pivers Island Road
Beaufort, North Carolina 28516

[2] National Marine Fisheries Service, NOAA
28 Tarzwell Drive Narragansett Laboratory
Narragansett, Rhode Island 02882

[3] Woods Hole Oceanographic Institution
Marine Research Facility 217, MS#50
Woods Hole, Massachusetts 02543

[4] Duke University Medical Center
P.O. Box 2628
Durham, North Carolina 27710

[5] David Clark Laboratory
North Carolina State University
P.O. Box 7617
Raleigh, North Carolina 27695-7617

[6] Marine Resources Research Institute
South Carolina Department of Natural Resources
P.O. Box 12559
Charleston, South Carolina 29422-2559

Abstract—The identification of sea bass (*Centropristis*) larvae to species is difficult because of similar morphological characters, spawning times, and overlapping species ranges. Black sea bass (*Centropristis striata)* is an important fishery species and is currently considered to be overfished south of Cape Hatteras, North Carolina. We describe methods for identifying three species of sea bass larvae using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) assays based on species-specific amplification of rDNA internal transcribed spacer regions. The assays were tested against DNA of ten other co-occurring reef fish species to ensure the assay's specificity. *Centropristis* larvae were collected on three cruises during cross-shelf transects and were used to validate the assays. Seventy-six *Centropristis* larvae were assayed and 69 (91%) were identified successfully. DNA was not amplified from 5% of the larvae and identification was inconclusive for 3% of the larvae. These assays can be used to identify sea bass eggs and larvae and will help to assess spawning locations, spawning times, and larval dispersal.

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A fundamental requirement of early life stage studies is the ability to identify individuals to species. Considerable knowledge of the eggs and larvae of most fish species in temperate and boreal ecosystems has been achieved (Kendall and Matarese, 1994; Berrien and Sibunka, 1999). In contrast, in subtropical and tropical ecosystems, the larvae of many species cannot be identified (Kendall and Matarese, 1994), even for groups of important fishery species. For example, of the 73 species in the snapper-grouper complex that range within U.S. federal waters, larvae of only one-half can be identified to species and eggs of most species are undescribed (Richards, 2006).

As anthropogenic stress on marine ecosystems continues to increase, new approaches to early life stage identification are needed to obtain critical information about exploited species and to improve the scientific basis for conservation. For example, immunological methods have been used successfully to identify invertebrate and fish larvae (Miller et al., 1991; Paugam et al., 2000; Garland and Zimmer, 2002; Taylor, 2004). Similarly, mitochondrial DNA sequences (Hare et al., 1994; Pegg et al., 2006) and restriction fragment length polymorphisms (RFLP) have been used to identify fish eggs, larvae, and adults (Daniel and Graves, 1994; Aranishi et al., 2005; Hyde et al., 2005). Though highly accurate, these methods have yet to be used widely as part of standard protocols for identifying fish larvae collected during research and monitoring surveys.

We examined the application of molecular techniques for identifying species within the genus *Centropristis* (sea bass). Five species of *Centropristis* are currently recognized in the western North Atlantic Ocean. In addition to black sea bass (*C. striata*), there are the following: bank sea bass (*C. ocyurus*), rock sea bass (*C. philadelphica*), twospot sea bass (*C. fusculа*), and *C. rufus.* Black sea bass is an important commercial and recreational species on the northeast and southeast U.S. continental shelf. Along

* Contribution number 635 of the South Carolina Marine Resources Center, Charleston, South Carolina 29422-2559.

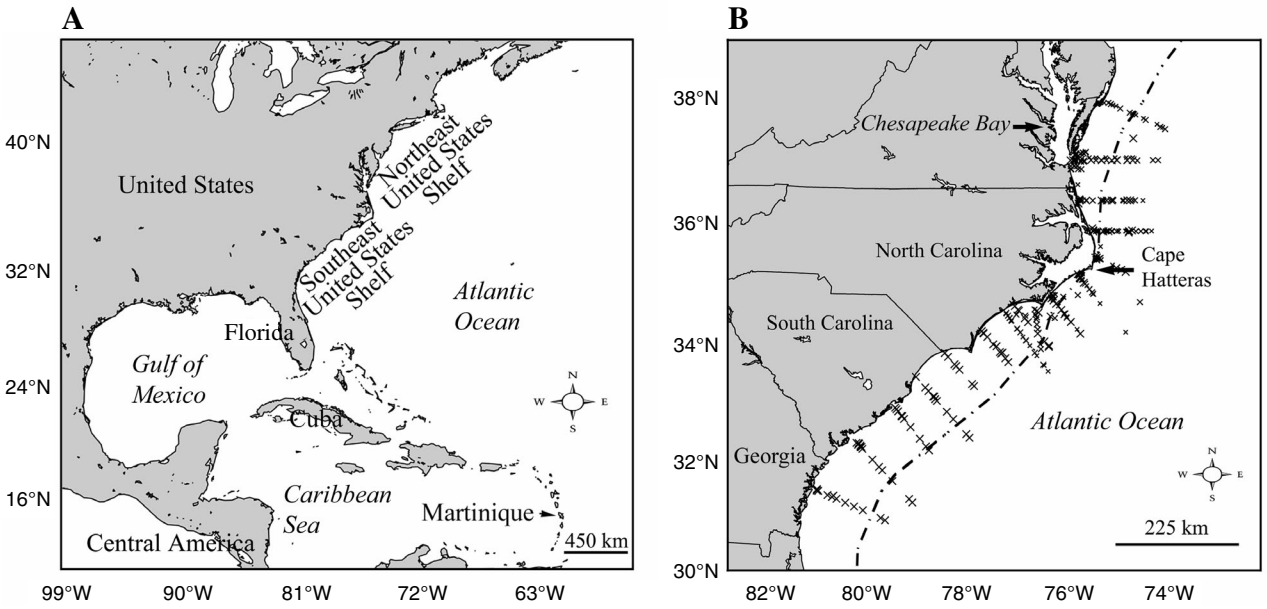

Figure 1

(**A**) Five species of *Centropristis* (sea bass) are currently recognized in the western North Atlantic Ocean. Black sea bass (*Centropristis striata*) ranges along the northeast and southeast shelves of the U.S. coast and inhabits the Gulf of Mexico on the northwest coast of Florida. Bank sea bass (*C. ocyurus*) and rock sea bass (*C. philadelphica*) are found along the southeast U.S. coast and in the Gulf of Mexico. *Centropristis rufus* is reported off Martinique in the southeastern Caribbean Sea. Twospot sea bass (*C. fuscula*) is reported from Cuba. (**B**) Sampling stations are marked with an x where *Centropristis* spp. larvae were collected during three cruises ranging from Chesapeake Bay to southern Georgia during September 2000, November 2000, and February through March 2001. During each cruise, transects running cross-shelf from 10 m to > 1000 m water depth were sampled. The dashed line approximates the 200-m depth contour.

the southeast U.S. shelf and the Gulf of Mexico where *C. striata*, *C. ocyurus*, and *C. philadelphica* cohabit (Fig. 1), larvae less than 12 mm cannot be reliably identified to species because fin ray formation is not complete. To identify larvae of these species, we developed species-specific polymerase chain reaction (PCR) and RFLP assays based on the internal transcribed spacer (ITS) regions of the ribosomal gene complex. The ITS regions are noncoding spacer regions situated between the 18S and 5.8S rRNA genes (ITS1) and between the 5.8S and 28S rRNA genes (ITS2) (Fig. 2A). The ITS regions work well for species identification because in most eukaryotic organisms these regions diverge rapidly during speciation, enabling even closely related species to be distinguished. The ITS sequences have been used to successfully identify species in groups as diverse as fungi (Lu et al., 2002), plants (Baldwin, 1992), insects (Rafferty et al., 2002), and dinoflagellates (Connell, 2001; Litaker et al., 2003). Other ITS-based assays have been developed to help address conservation and management problems in the trade of shark products (Chapman et al., 2003; Shivji et al., 2005) and for identifying wildlife tissues in forensic applications (Sweijd et al., 2000; Ambercrombie et al., 2005).

PCR and RFLP assays offer rapid methods for identification of fish larvae and eggs. The PCR assays can be performed in a one-step process after DNA extraction and they enable species identifications to be made even when meristic characters and other identifying features have been damaged or are underdeveloped. Alternatively, the RFLP assays provide a cost efficient, two-step, PCR-based species identification method. In the first step, PCR is used to amplify the ITS regions. In the second step, restriction enzyme digestion of the PCR product is conducted and species-specific fragments are analyzed. This study describes the development, optimization, and validation of PCR and RFLP assays for three species of *Centropristis*.

Materials and methods

Collection and preservation of adult fish

Tissue samples were obtained from adult and juvenile fish and either stored in 95% ethanol or frozen at –80°C. *Centropristis striata* were collected from the Atlantic coast of the southeast United States, and *C. ocyurus* and *C. philadelphica* were collected from both the southeastern U.S Atlantic coast and the Gulf of Mexico. At least three individuals from each location were analyzed. Ten other co-occurring southeast U.S. reef fishes were also collected for sequencing of the ITS regions and served as negative controls for the PCR and RFLP assays. Five of these species were from the subfamily Serraninae: sand perch (*Diplectrum formosum*), butter hamlet (*Hypoplec-*

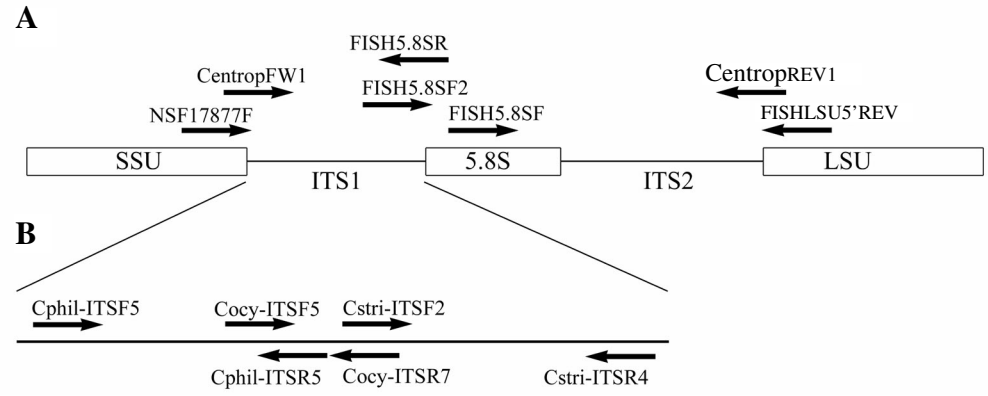

Figure 2

Diagram of the small subunit (SSU), internal transcribed spacer regions (ITS), 5.8S, and large subunit (LSU) genes. (**A**) Location of the universal polymerase chain reaction (PCR) primers used to amplify the ITS spacer regions, the genus-specific restriction fragment length polymorphism (RFLP) primers, and the internal sequencing primers. (**B**) Location in the ITS1 region of the *Centropristis* PCR assay primers.

trus unicolor), pygmy sea bass (*Serraniculus pumilio*), tattler (*Serranus phoebe*), and belted sandfish (*Serranus subligarius*). Four species were from the family Serranidae, red grouper (*Epinephelus morio*), rock hind (*Epinephelus adscensionis*), speckled hind (*Epinephelus drummondhayi*), gag grouper (*Mycteroperca microlepis*), and one from a related perciform family, Sparidae, the spottail pinfish (*Diplodus holbrooki*).

Collection and preservation of larvae

Three cruises were conducted from Chesapeake Bay to southern Georgia during early fall (September 2000), late fall (November 2000), and late winter (February and March 2001). During each cruise, transects running cross-shelf from 10 m to >1000 m water depth were sampled (Fig. 1). The deepest stations were sampled to 50 m. Ichthyoplankton were collected with a 1-m Tucker trawl fitted with 333-μm mesh nets. The Tucker trawl was fitted with three nets. The first net sampled from the surface to the deepest deployment depth. For stations in <50 m water depth, the lower and upper halves of the water column were sampled discretely by using the second and third nets during the retrieval of the Tucker trawl. For stations in >50 m water depth, the 50–25 m depth was sampled discretely with the second net and the 25–0 m depth interval was sampled discretely with the third net. Depth was determined from the wire angle of the gear and length of wire deployed. Tow speed was between 1.5 and 2 knots.

Samples were initially preserved in 95% ethanol. All larvae were sorted from these samples, transferred to 70% ethanol, and identified to the lowest taxonomic level possible. Larvae were identified to the genus *Centropristis* based on characteristic body shape, pigmentation, and when available, dorsal and anal fin meristics (Kendall, 1972). Seventy-six larvae were chosen for genetic analyses. These specimens were selected in an attempt to analyze equal numbers of larvae collected from north and south of Cape Hatteras, North Carolina. In this respect, the larvae were not a random sample of the *Centropristis* larvae identified from the collections. The larvae ranged in size from 1.5–11 mm notochord length (or standard length). Each individual to be used for PCR analyses was rinsed three times with clean 95% ethanol, digitally imaged, and stored in a separate sterile vial with 95% ethanol. To prevent DNA cross-contamination, the forceps used for handling larval fish were decontaminated between specimens with 10% sodium hypochlorite, rinsed with clean deionized water, and dried with a clean Kimwipe (Kimberly-Clark, Roswell, GA).

Determination of ITS region sequences

To identify species-specific primer sites it was first necessary to amplify and sequence the ITS regions from adult *Centropristis* and other reef fish. To accomplish this, universal PCR primers were designed for the amplification of the 3′ end of the small subunit (SSU), the internal, transcribed spacer region (ITS1), 5.8S subunit (5.8S), the second internal transcribed spacer region (ITS2), and the first ~48 base pairs of the large subunit (LSU) rRNA genes (~1300-bp product). The universal forward primer NSF1787F (Fig. 2A, Table 1) was designed with the reverse complement of the universal reverse primer, NSR1787/18, listed on the ribosomal RNA database website. The universal reverse primer FishLSU5'Rev (Fig. 2A, Table 1) was designed by aligning (CLUSTAL X algorithm; Thompson et al., 1997) the large subunit rDNA sequences of the following fish species that were available in GenBank: European perch (*Perca fluviatilis* Z18686), rainbow trout (*Oncorhynchus*

mykiss OMU34341, Z18683), tub gurnard (*Trigla lucerna* Z18768), Atlantic mackerel (*Scomber scombrus* Z18693), zebrafish (*Brachydanio rerio* AJ306603), *Pimelodella cristata* (AJ306596), Atlantic herring (*Clupea harengus* Z18764), dab (*Limanda limanda* Z18681), brown bullhead (*Ictalurus nebulosus* Z18678), spotted green pufferfish (*Tetraodon nigroviridis* AJ270038, AJ270037), common carp (*Cyprinus carpio* AF133089), black ghost (*Apteronotus albifrons* AJ306595), Eurasian minnow (*Phoxinus phoxinus* AJ306604), *Synodontis clarias* (AJ306597), *Orinocodoras eigenmanni* (AJ306606), angler (*Lophius piscatorius* Z18765), European pilchard (*Sardina pilchardus* Z18767), coelacanth (*Latimeria chalumnae* U34336), ghost shark (*Callorhinchus milii* AY049812), Florida gar (*Lepisosteus platyrhynchus* Z18680), arawana (*Osteoglossum* sp. Z18684), rabbit fish (*Chimaera monstrosa* Z18674), European eel (*Anguilla anguilla* Z18673), bowfin (*Amia calva* Z18672), and browneye skate (*Raja schmidti* AF278683).

DNA purification

For adult specimens, approximately 50–100 mg of muscle tissue or a clipping from the fin was collected from either frozen or ethanol-preserved specimens and DNA was extracted with a Roche High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's protocol for isolation of nucleic acids from mammalian tissue. The one exception to the manufacturer's protocol was that we used a volume of 50 μL to elute the DNA in the final step of the procedure. For ethanol-preserved specimens, the tissues were rehydrated in three changes of sterile tris ethylenediaminetetraacetic acid (TE) buffer for 30 minutes each. Tissues were then transferred to the cell lysis solution, and DNA was purified following the manufacture**'s** protocol.

DNA extraction from larval fish was accomplished by first placing them in separate sterile 55 mm Petri dishes and allowing them to rehydrate in sterile TE buffer [10mM Tris HCl (pH 7.4), 1mM disodium ethylenediaminetetraacetate (EDTA) (pH 8.0)] for 30 minutes. Next, an eye was microdissected with fine forceps and the DNA was purified as described above. This limited the physical damage to the larvae, allowing them to be used for future meristic studies. The forceps used for microdissection were decontaminated between specimens as described above.

PCR amplification, cloning, and sequencing procedures for ITS regions of adult fish

The PCR amplification reaction mixture used to amplify adult fish ITS regions is listed in Table 2 . DNA was PCR amplified in a MJ Research MiniCycler (MJ Research, Waltham MA) under the following touchdown cycling conditions: 2 min. at 95°C, 35 cycles each consisting of 40 sec. denaturation at 95°C, 40 sec. initial annealing temperature at 64°C which decreased by 0.5°C per cycle for six cycles and 61°C thereafter and an extension of 1.5 min. at 72°C. This procedure was followed by a final extension of 5 min. at 72°C. A 4-μL aliquot of each PCR reaction was checked for the presence of a specific amplification product by agarose gel electrophoresis (2% agarose, tris acetate EDTA [TAE gel], 50 volts) and ethidium bromide staining. PCR reactions containing specific products were cloned into the plasmid vector pCR2.1 by using the Topo TA Cloning Kit and by following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Plasmids were isolated and purified with a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and sequenced with an ABI377 DNA sequencer employing the Deoxy Terminator Cycle sequencing kit (Applied Biosystems–ABI, Foster City, CA). In addition to the ten local reef fishes, three adult fish of each *Centropristis* species were PCR amplified and cloned. DNA templates were sequenced completely in both directions with the M13F, M13R, Fish5.8SF, Fish5.8SF2, and Fish5.8SR primers (Fig. 2A, Table 1). The resulting 3′ SSU to 5′ LSU sequence for each species was assembled by using the Vector NTI program (Informax Inc., Bethesda, MD) and submitted to GenBank (EF472464–EF472500).

Table 1

Primers used in this study to amplify and sequence the 3′ end of the small subunit gene, internal transcribed spacer 1, 5.8S gene, internal transcribed spacer 2, and the first approximately 48-bp of the large subunit gene

Primer name	Sequence (5′–3′)
Forward sequencing	
M13F (plasmid vector)	GTAAAACGACGGCCAG
NSF1787F (universal forward)	CCGTAGGTGAACCTGCGG
Fish5.8SF	AGCTGCGAGAACTAATGTGAA
Fish5.8SF2	TGCTCTGCTCGGGCTGTAGCG
Reverse sequencing	
M13R (plasmid vector)	CAGGAAACAGCTATGAC
FishLSU5'Rev (universal reverse)	CTTAAATTCAGCGGGTTGTCT
Fish5.8SR	TTCACATTAGTTCTCGCAGCT

Phylogenetic analysis

The ITS rDNA gene sequence from *C. striata*, *C. ocyurus*, *C. philadelphica*, and *Diplectrum formosum* (for an outgroup) were aligned using the CLUSTAL-X algorithm (Thompson et al., 1997). A Bayesian phylogenetic analysis of the aligned sequences was performed with the MrBayes 3.1 program (Huelsenbeck et al., 2001). Posterior probabilities were calculated by using a Metropolis-coupled Markovian Chain Monte Carlo approach and with sampling conducted according to the Metropolis–

Table 2

Polymerase chain reaction (PCR) reagent concentrations used amplify fish internal transcribed spacer regions and conduct PCR and restriction fragment length polymorphism (RFLP) assays for *Centropristis striata* (black sea bass), *C. ocyurus* (bank sea bass), and *C. philadelphica* (rock sea bass).

Reagent	Fish ITS PCR reaction mix	*C. striata* assay	*C. ocyurus* assay	*C. philadelphica* assay	RFLP PCR reaction mix
Tris-HCl	100 mM, pH 8.8	100 mM, pH 8.8	200 mM, pH 8.4	100 mM, pH 8.8	100 mM, pH 8.3
$MgCl_2$	15 mM	15 mM	30 mM	15 mM	15 mM
KCl	750 mM	750 mM	500 mM	750 mM	750 mM
Primer I	25 pmoles	25 pmoles	25 pmoles	25 pmoles	25 pmoles
Primer II	25 pmoles	25 pmoles	25 pmoles	25 pmoles	25 pmoles
dNTP mix	10 mM	10 mM	10 mM	10 mM	10 mM
DMSO	5%	5%	5%	5%	5%
Taq DNA polymerase	0.25 units	0.25 units	0.25 units	0.25 units	0.25 units
DNA template	20–50 ng	20–50 ng	20–50 ng	20–50 ng	20–50 ng
Reaction volume	50 μL	50 μL	50 μL	50 μL	50 μL

Table 3

Primer pairs used to conduct the *Centropristis* (sea bass) polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) assays. bp=base pairs.

Species	Primer	Primer sequence 5′–3′	PCR product size
C. striata assay	Cstri-ITSF2 (forward) Cstri-ITSR4 (reverse)	TGGACCGGCTTTCCTCCCG CAATGAGGGTTGGAGAAAGGG	230 bp
C. ocyurus assay	Cocy-ITSF5 (forward) Cocy-ITSR7 (reverse)	CTCGTCCTCCTTGCGGTGG GGAGGTTTTGGTTTGTGTAGG	158 bp
C. philadelphica assay	Cphil-ITSF5 (forward) Cphil-ITSR5 (reverse)	CACTGCCACTGCCTCCAC ACGGAGCCAGCTTTCACC	238 bp
Centropristis RFLP assay	CentropFW1 (forward) CentropREV1 (reverse)	GATCATTACCGGTCGGTTGC GTAGTCGAAAAGTGGAGGCAG	~1200 bp

Hastings algorithm. The phylogenetic analysis employed two concurrent analyses of four chains each. In each analysis, one cold and three incrementally heated chains were used where the heat of the ith chain is $B = 1/[1+(i-1)T]$ and $T = 0.2$. Initial phylogenetic trees for each chain were random and used default starting values of MrBayes. A single run consisting of 100,000 generations was conducted. The run was sampled at every 100th tree. Only trees sampled after a stable burn-in of 1000 generations were used. The results were plotted as a rooted phylogram with *Diplectrum formosum* as the outgroup. An outgroup is any species occurring outside a particular branch or clade, i.e., a species that is further towards the root of a phylogenetic tree.

Development of species-specific PCR assays for identifying larval fish

The 3′ SSU – 5′ LSU sequences for *Centropristis striata* (EF472473-EF472481), *C. philadelphica* (EF472482-EF472490), *C. ocyurus* (EF472491-EF472499), *Diplectrum formosum* (EF472472), *Diplodus holbrooki* (EF472471), *Epinephelus morio* (EF472468), *Epinephelus adscensionis* (EF472470), *Epinephelus drummondhayi* (EF472469), *Hypoplectrus unicolor* (EF472467), *Mycteroperca microlepis* (EF472466), *Serraniculus pumilio* (EF472465), *Serranus phoebe* (EF472464), and *Serranus subligarius* (EF472500) were aligned by using the CLUSTAL-X algorithm. The alignments were used to identify unique ITS sequences and to develop species-specific PCR assays. For all three *Centropristis* assays, we used forward and reverse primers located within the ITS1 region (Fig. 2B). The PCR primers used for each assay are listed in Table 3. The assays were conducted under touchdown PCR cycling conditions with an MJ Research PTC-150 MiniCycler. The PCR reaction profile for the *C. striata* assay was as follows: 2 min. at 95°C, 35 cycles each consisting of 30 sec. denaturation at 95°C, 40 sec. initial annealing temperature at 66°C which decreased by 0.5°C per cycle for four cycles and 64°C thereafter, and

an extension of 45 sec. at 72°C. This was followed by a final extension of 5 min. at 72°C. The cycling conditions for the *C. ocyurus* assay were the following: 2 min. at 95°C, 35 cycles each consisting of 30 sec. denaturation at 95°C, 40 sec. initial annealing temperature at 64°C which decreased by 0.5°C per cycle for four cycles and 62°C thereafter and an extension of 45 sec. at 72°C. This was followed by a final extension of 5 min. at 72°C. Lastly, the cycling conditions for the *C. philadelphica* assay were: 2 min. at 95°C, 35 cycles each consisting of 30 sec. denaturation at 95°C, 40 sec. initial annealing temperature at 60°C which decreased by 0.5°C per cycle for four cycles and 58°C thereafter and an extension of 45 sec. at 72°C. This was followed by a final extension of 5 min. at 72°C. An aliquot (4 μL) from each amplification was analyzed by agarose gel electrophoresis (3% agarose TAE gel, 75V). The sizes of the PCR products were estimated with either a 123-bp or 50-bp ladder (Promega, Madison, WI).

Validation of the PCR assays for identifying larval fish

Following assay development, the primer pairs were tested for cross-reactivity with a panel of DNAs that included the 10 reef fish species listed previously (20–50 ng of DNA per PCR reaction). DNA from 76 *Centropristis* larvae was extracted using the methods described above. The larvae were assayed by using 20–50 ng of DNA from each specimen. Each PCR assay included a positive control, a negative control, a blank DNA extraction control, and two PCR inhibition controls. The positive control incorporated 20 ng of appropriate *Centropristis* DNA in the reaction mixture. The negative control substituted 1×PCR buffer for DNA to confirm that the reagents were not contaminated with target DNA. The blank extraction control was included to assess possible cross-contamination during the extraction procedures. The inhibition control consisted of spiking 20 ng of appropriate *Centropristis* DNA into two arbitrarily chosen larval fish DNA samples. The PCR inhibition controls confirmed that negative PCR reactions were due to the absence of appropriate *Centropristis* DNA and not from PCR inhibition. The *C. striata* and *C. ocyurus* assays were tested on all of the larvae in the collection. The *C. philadelphica* assay was applied by process of elimination to larvae that did not yield results from the *C. striata* or *C. ocyurus* assays.

Development of RFLP assays for identifying larval fish

Forward and reverse genus-specific primers for three species of *Centropristis* were designed from the 3′ SSU–5′ LSU sequence alignment described above. The forward primer, CentropFW1 (Table 3), overlapped the boundary of the 3′ end of the SSU rRNA gene and the 5′ end of the ITS1 spacer (Fig. 2A). The reverse primer, CentropRev1 (Table 3), overlapped the boundary of the 3′ end of the ITS2 spacer and the 5′ end of the LSU rRNA gene (Fig. 2A). The primer pair yielded an approximate 1200-bp PCR product and was tested for cross-reactivity against the 10 non-*Centropristis* reef fishes listed above. The RFLP-PCR reaction mix is listed in Table 2. Twenty to 50 ng of genomic DNA was used per PCR reaction. PCR amplifications were conducted with an MJ Research PTC-150 MiniCycler under the following cycling conditions: 2 min. at 95°C, 40 cycles each consisting of 30 sec. denaturation at 95°C, 40 sec. initial annealing temperature at 63°C which decreased by 0.5°C per cycle for six cycles and 60°C thereafter, and an extension of 1 min. at 72°C. This was followed by a final extension of 5 min. at 72°C. A 4-μL aliquot of each PCR reaction was checked for the presence of a specific amplification product by agarose gel electrophoresis (2% agarose TAE gel, 50 V) and ethidium bromide staining. The sizes of the PCR products were estimated by using the DNA molecular weight marker IX (Roche Diagnostics, Indianapolis, IN).

RFLP analysis of *Centropristis* PCR products was simulated with the RFLP analysis tool in the Vector NTI program (Informax Inc., Bethesda, MD) to identify species-specific RFLP patterns. The restriction enzyme *Alu* I (New England Biolabs, Beverly, MA) was selected on the basis of the distinct restriction patterns predicted for each *Centropristis* species. Restriction digests were performed in 30-μL reactions containing 3 μL of New England Biolabs 10× reaction buffer #3, 25 μL of PCR product, 2000 units of *Alu I* enzyme, and were incubated at 37°C for 2 hours. Restriction fragments were separated by gel electrophoresis on 3% agarose, Tris-Borate EDTA NuSieve 3:1 gels (Cambrex Bio Science Rockland, Inc., Rockland, ME) and fragments sizes were estimated by using a 50-bp DNA ladder (Promega, Madison, WI).

Results

Species-specific molecular assays based on differences in the ITS rDNA regions were developed successfully for the identification of *Centropristis* larvae. The approach we employed in developing the molecular assays was 1) to identify universal PCR primer sites that would amplify the ITS regions of many fish species; 2) to sequence the ITS regions from three *Centropristis* species and other co-occurring reef species; 3) to develop species-specific PCR and RFLP assays for the *Centropristis* species based on alignments of the resulting sequences; and 4) to use the assays to identify field-collected larval *Centropristis*.

The universal PCR primers (Table 1) were successful in amplifying the ITS regions of many reef fish species. The primers generated an approximate 1200–1300 base pair product that varied with the species. A phylogenetic analysis of the *Centropristis* ITS sequence alignments showed that between-species sequence divergence in this region was much greater than within-species divergence (Fig. 3). This variation made it possible to develop species-specific PCR assays for *C. striata*, *C. ocyurus*, and *C. philadelphica*. The primers were tested for cross-reactivity against ten other related reef fish

species and they did not amplify non-target species (Fig. 4). The accuracy of the species-specific assays was checked by PCR assaying 15 adult and juvenile sea basses (5 of each species). The PCR assays were successful in identifying the target species and did not cross-react among the three *Centropristis* species.

We validated the PCR assays by using them to assess the identity of 76 *Centropristis* larvae (Fig. 5). Sixty-nine of the 76 larvae (91%) were identified successfully: 33 *C. striata*, 32 *C. ocyurus*, and four *C. philadelphica*. DNA from four of the larvae failed to PCR amplify and in three cases the PCR assay results were inconclusive. The four DNA samples that failed to amplify were tested for PCR inhibition by spiking a small amount of target *Centropristis* plasmid DNA into PCR reaction mixes containing DNA from the larvae. These spiked controls all amplified, indicating that PCR inhibition was not a factor. DNA was re-extracted from the larvae and attempts were made to re-amplify it with the species-specific primers and the universal primers. Neither primer sets produced a PCR product, indicating that DNA degradation had occurred to the extent that amplifiable DNA could not be isolated from these four specimens.

The three inconclusive results were symptomatic of DNA cross-contamination between larval samples. Both the *C. striata* and *C. ocyurus* specific primer sets produced correct size PCR products for these samples. DNA from these larvae was re-extracted in an attempt to eliminate the cross-contamination, but multiple PCR assay attempts yielded the same ambiguous results. The issues of cross-contamination and methods to avoid it will be discussed below.

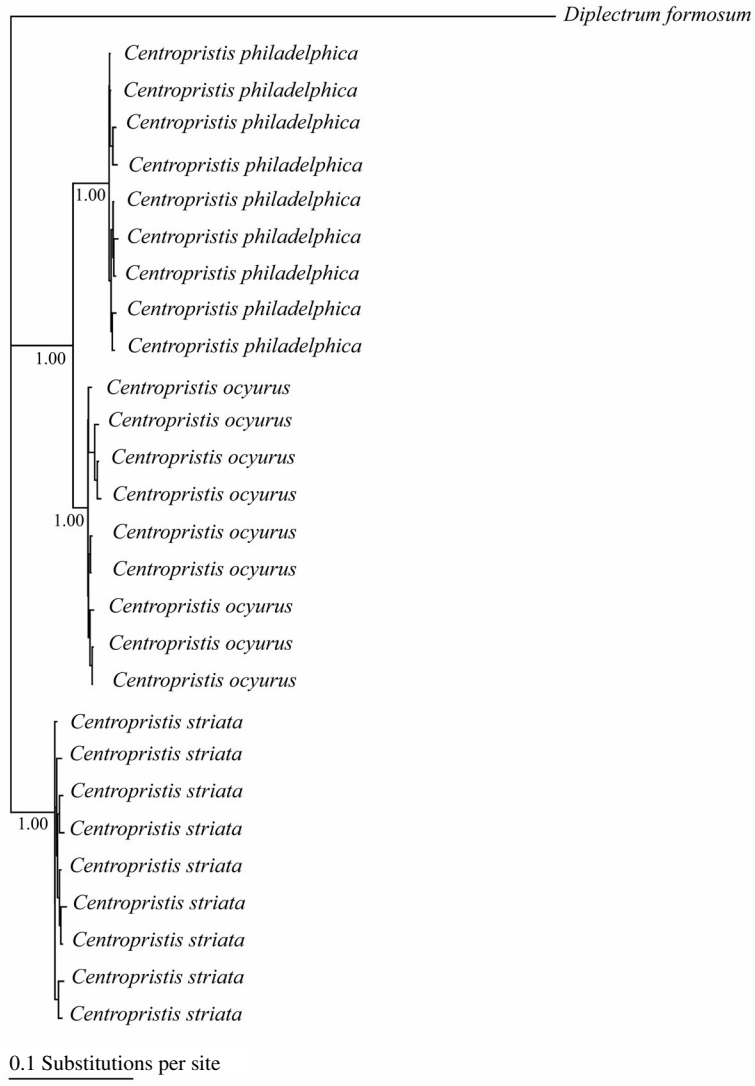

Figure 3

Phylogeny showing the relationship between black sea bass (*Centropristis striata*), bank sea bass (*C. ocyurus*), and rock sea bass (*C. philadelphica*) based on ITS1, 5.8S, and ITS2 rDNA sequences. Sand perch (*Diplectrum formosum*) served as the outgroup. Branch support for each species was 100% (1.0). The scale at the bottom of the phylogeny is proportional to the branch lengths in the phylogenetic tree that correspond to a divergence of 0.1 nucleotide substitutions per base pair in the DNA sequences. The within-species sequence variation was consistently less than that observed between species.

RFLP assays were also developed for each species as an alternative to the PCR assays. Genus-specific PCR primers (Table 3) that flanked the ITS regions were used to amplify DNA from identified adult sea basses. The genus-specific primer sites were located very near the universal primer sites but were designed to amplify only *Centropristis* DNA. The primers were tested for cross-reactivity against the same related reef species that were used in the PCR assays. Cross-reactivity did not occur (Fig. 6A). The PCR products were digested with the *Alu* I restriction enzyme. Electrophoresis yielded unique banding patterns (Fig. 6B). Digests of *C. striata* yielded fragments of 446, 418, 218, 125, and 4 base pairs. The restriction fragment sizes for *C. ocyurus* were 449, 362, 245, 120, 51 bp, and 4 base pairs. For *C. philadelphica* the fragments were 481, 261, 187, 185, 121, 58, and 4 base pairs. The advantages and disadvantages of this diagnostic approach are discussed below.

Discussion

Ribosomal DNA-specific assays for *Centropristis* larvae have proven to be robust and accurate. The difficul-

ties associated with identifying *Centropristis* larvae prompted this work. The ITS region was chosen for assay development because it diverges rapidly during speciation and contains unique primer sites for distinguishing species (Baldwin, 1992; Lu et al., 2002; Litaker et al., 2003). The divergence between *Centropristis* species was supported by the ITS phylogenetic analysis, which showed that *C. striata, C. ocyurus,* and *C. philadelphica* were genetically distinct from each other and from another related serranid species, *Diplectrum formosum* (Fig. 3). The analysis further showed that *C. ocyurus* and *C. philadelphica* are more closely related when compared to *C. striata,* which is consistent with the morphological characteristics of the adult fish. One of the goals of this study was to conduct the larval assays in a manner that would cause minimal damage to the preserved specimens. The method used in our study, where DNA was recovered from a single eye, made it possible to perform morphometric analyses on genetically identified fish and to evaluate whether species-specific characteristics exist that can be used to identify the larvae.

The PCR assays were successfully validated by using larvae that were collected on cruises ranging from Chesapeake Bay to southern Georgia during September 2000, November 2000, and February through March 2001 (Fig. 1). The peak spawning season for *C. striata* occurs between June and September in the Mid-Atlantic Bight (Able et al., 1995) and between March and May in the South Atlantic Bight (Wenner et al., 1986; Mercer, 1989; McGovern et al., 2002). Data for spawning of *C. philadelphica* comes from Miller (1959) who concluded that spawning occurs during May and June. Manooch (1984) indicated that ripe females of *C. ocyurus* were collected in the South Atlantic Bight during March and April and that young fish appear in late April, suggesting that spawning occurs offshore in spring. Results from our study confirmed that spawning of *C. ocyurus* overlapped with that of *C. striata* and occurred in September and as early as February to March. The data from our study for *C. philadelphica* are limited, making it difficult to determine whether the low abundance was due to spawning period, spawning locations, or spawning stock biomass. *Centropristis philadelphica* larvae may have been less abundant in the collections because most of the sampling was conducted in waters deeper than 10 m. Trawling studies off the southeastern United States indicate that *C. philadelphica* is a year-round resident of shallow coastal waters (<10 m) with sandy-mud substrates (Wenner and Sedberry, 1989) and is not present at greater depths over sandy-mud or hard bottoms (Wenner et al., 1979; Sedberry and Van Dolah, 1984). Furthermore, it is likely that the sampling of larvae did not occur during the peak spawning season for *C. philadelphica* (Miller, 1959).

Because PCR is a highly sensitive molecular technique, care must be taken to avoid contamination. The data indicated that DNA cross-contamination occurred in three of the larval samples. We hypothesize that the DNA contamination occurred while the *Centropristis* larvae were stored collectively or during the sorting of the larvae. Field-collected larvae should therefore be preserved individually and utensils or tools used to process larvae should be decontaminated between larvae during the sorting process with a reagent that destroys DNA.

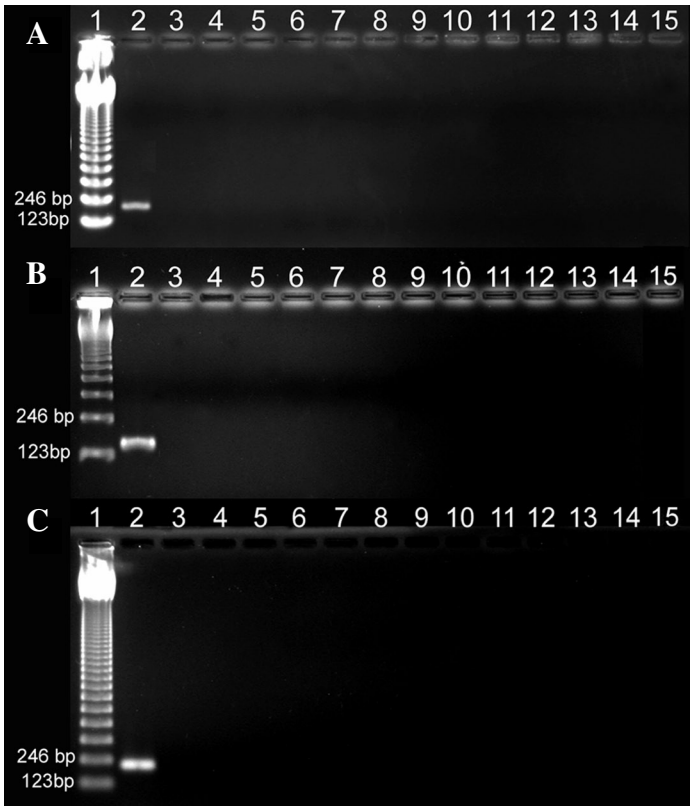

Figure 4

Results of cross-reactivity tests for (**A**) black sea bass (*Centropristis striata*), (**B**) bank sea bass (*C. ocyurus*), and (**C**) rock sea bass (*C. philadelphica*) polymerase chain reaction (PCR) assays. In each panel, the gels were loaded in the following order: lane 1, 123-bp ladder; lane 2, positive controls for A) *C. striata*, B) *C. ocyurus*, and C) *C. philadelphica*; lane 3, A) *C. ocyurus,* B) *C. striata*, C) *C. philadelphica*; lane 4, A) *C. philadelphica*, B) *C. striata*, C) *C. ocyurus*; lanes 5–14, sand perch (*Diplectrum formosum*), spottail pinfish (*Diplodus holbrooki*), red grouper (*Epinephelus morio*), rock hind (*Epinephelus adscensionis*), speckled hind (*Epinephelus drummondhayi*), butter hamlet (*Hypoplectrus unicolor*), gag grouper (*Mycteroperca microlepis*), pygmy sea bass (*Serraniculus pumilio*), tattler (*Serranus phoebe*), and belted sandfish (*Serranus subligarius*); lane 15, no DNA control.

PCR versus RFLP assays

The species-specific PCR and RFLP assays can be used to accurately identify larvae. The decision of which assay to use could be based on labor and reagent costs. The PCR assays are attractive because they are rapid single step assays, whereas the RFLP assays employ PCR in the first step and restriction enzyme digestion of the PCR products in the second step. In most cases, PCR reagents are more expensive than those needed for restriction enzyme analysis. However, PCR assays can be cost effective if they are multiplexed. Excellent examples of successful multiplex PCR assays are described by Chapman et al. (2003) and Hyde et al. (2005). Unfortunately our attempts at multiplexing the assays were unsuccessful because of the production of nonspecific PCR products.

RFLP assays are cost effective because they require less expensive reagents than PCR assays. However, the RFLP assays require an extra hour or two to process and involve additional labor costs. The RFLP assays developed here employed genus-specific primers rather than species-specific primers to amplify *Centropristis* genomic DNA from adult fish (Table 3). Using genus-specific primers, we simplified the PCR reaction mixtures for all three species and ensured that only *Centropristis* DNA would generate PCR products. The resulting PCR products were subsequently digested with *Alu* I enzyme to produce species-specific fragments that were easily distinguished by their unique banding patterns once electrophoretically separated on an agrose gel. The unique sizes of these fragments were due to species-specific differences in the locations of the *Alu* I restriction sites within the ITS1, 5.8S gene, and ITS2 regions (Fig. 6B).

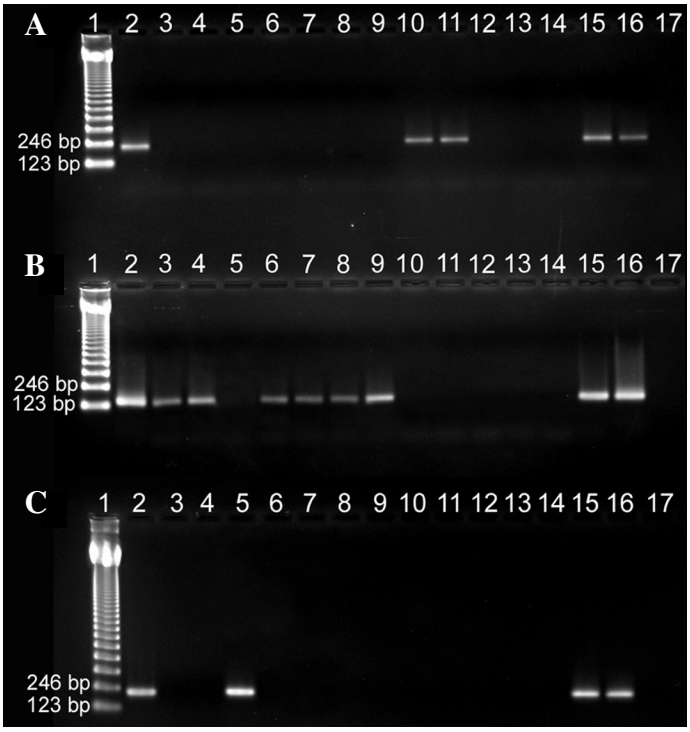

Figure 5

Results of species-specific polymerase chain reaction (PCR) assays for sea bass (*Centroprisitis*) larvae (**A**) black sea bass (*Centropristis striata*), (**B**) bank sea bass (*C. ocyurus*), and (**C**) rock sea bass (*C. philadelphica*). In each panel, the gels were loaded in the following manner: lane 1, 123-bp ladder; lane 2, positive controls for A (*C. striata*), B (*C. ocyurus*), and C (*C. philadelphica*); lanes 3–13, *Centropristis* larvae, lane 14, negative control; lane 15–16, PCR inhibition controls; lane 17, no DNA control.

Shipboard operational molecular assays

The PCR and RFLP assays could be conducted at sea with a small thermocycler and the other minor equipment necessary for DNA extraction. If onboard conditions were favorable, the assays could be analyzed immediately after PCR thermocycling by gel electrophoresis and with the use of a digital imaging camera. Similar methods described by Hyde et al., (2005) were used at sea to identify the eggs and larvae of blue marlin (*Makaira nigricans*), shortbill spearfish (*Tetrapturus angustirostris*), and wahoo (*Acanthocybium* solandri). Their procedures incorporated a boiling method to quickly prepare amplifiable DNA from fresh tissue. If this would not be practical, then PCR products could be frozen and analyzed at an onshore laboratory. Results for 30 larval assays could be attained in four hours or less at a cost of ~$5 per sample, excluding the cost of equipment. Alternatively, larvae could be collected and sorted; all *Centropristis* larvae would be separated and assayed or stored individually in 95% ethanol for processing at a shore-based laboratory. DNA could then be extracted from a small tissue sample (e.g., an eye) from each specimen. The intact larvae would be returned to storage in 95% ethanol in case any additional morphological or molecular evaluation was required. If analysis of a large number of samples was desired, the individual PCR assays could be adapted to a SYBR green quantitative PCR format. Because this approach eliminates the need for gel electrophoresis, it could be used to process hundreds of samples provided that sorting could be expedited.

Conclusion

Both the species-specific PCR and RFLP assays described in this article can successfully identify *C. striata*, *C. ocyurus*, and *C. philadelphica*. The assays are rapid, cost effective, simple to perform, and highly accurate.

Furthermore, these techniques can be integrated with environmental and oceanographic data to allow almost immediate investigation of spawning times, spawning locations, and larval dispersal. The ability to identify *Centropristis* larvae to species throughout their range will provide a foundation for future studies where early life history stages of fishes are used to investigate questions related to fisheries management. This study also provides a model for the future development of species-specific assays for other commercially important fish species.

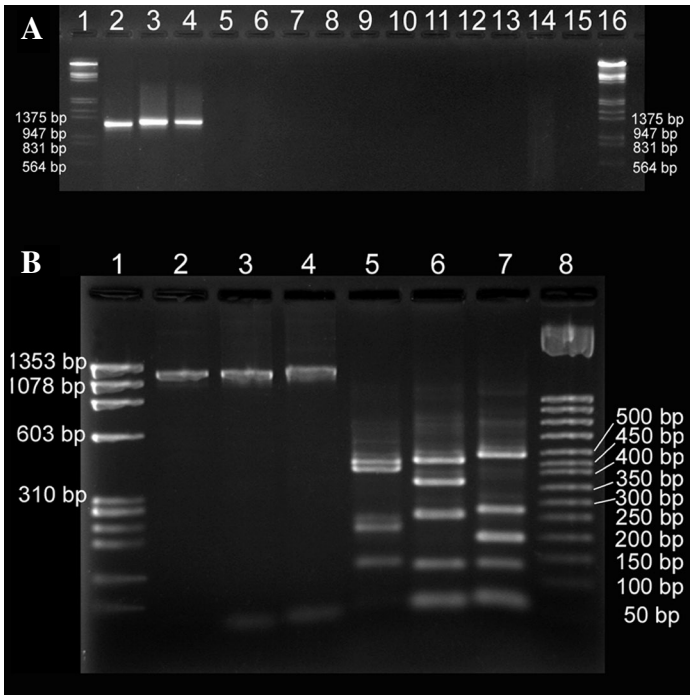

Figure 6

(**A**) Cross-reactivity test of genus-specific polymerase chain reaction (PCR) primers (CentropFW1 and CentropRev1) used for restriction fragment length polymorphism analysis (RFLP) of black sea bass (*Centropristis striata*), bank sea bass (*C. ocyurus*), and rock sea bass (*C. philadelphica*). The gel was loaded in the following order: lanes 1 and 16, DNA molecular weight marker III (Roche Diagnostics, Indianapolis, IN); lane 2, *C. striata*; lane 3, *C. ocyurus*; lane 4, *C. philadelphica*; lanes 5–14, sand perch (*Diplectrum formosum*), spottail pinfish (*Diplodus holbrooki*), red grouper (*Epinephelus morio*), rock hind (*Epinephelus adscensionis*), speckled hind (*Epinephelus drummondhayi*), butter hamlet (*Hypoplectrus unicolor*), gag grouper (*Mycteroperca microlepis*), pygmy sea bass (*Serraniculus pumilio*), tattler (*Serranus phoebe*), and belted sandfish (*Serranus subligarius*); lane 15, no DNA control. (**B**) PCR-RFLP analysis of *Centropristis* ITS1, 5.8S, and ITS2 rDNA amplified with genus-specific primers described in the text. Each PCR product was digested with the restriction enzyme *Alu* I. Lane 1, DNA molecular weight marker IX (Roche Diagnostics, Indianapolis, IN); lanes 2–4 PCR products for *C. striata*, *C. ocyurus*, and *C. philadelphica*; lanes 5–7, *Alu* I digests of PCR products for *C. striata, C. ocyurus*, and *C. philadelphica*; lane 8, 50-bp ladder.

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