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Multiplex assay to identify eggs of three fish species from the northern Gulf of Mexico, using locked nucleic acid Taqman real-time PCR probes

Keith M. Bayha[1,2,*], William M. Graham[1], Frank J. Hernandez Jr.[1]

[1]Dauphin Island Sea Lab, 101 Bienville Blvd., Dauphin Island, Alabama 36528, USA
[2]*Present address:* University of California Merced, School of Natural Sciences, 5200 North Lake Rd., Merced, California 95348, USA

ABSTRACT: Understanding the early life histories of economically and ecologically important fish species is vital to developing effective management strategies. While adult-derived egg production estimates are important to fisheries management models, determining fish egg abundance in the wild is cumbersome and oftentimes impossible, as most fish eggs cannot be morphologically identified to species. Here, we report on the development of a real-time polymerase chain reaction assay to identify the eggs of 3 important Gulf of Mexico fish species (red drum *Sciaenops ocellatus*, red snapper *Lutjanus campechanus* and vermilion snapper *Rhomboplites aurorubens*). This single-tube, single-egg assay uses 3 species-specific fluorescently labeled locked nucleic acid (LNA) Taqman probes to identify DNA extracted from individual eggs of the 3 targeted species. To ensure that the probes were truly species specific, the LNA Taqman assay was tested against DNA from 25 or more known adults of each target species as well as DNA from 62 non-target northern Gulf of Mexico fishes. All DNA extracts from the 3 target species showed the appropriate positive reaction from the assay, while all 62 other DNA extracts showed null reactions. The assay was also tested individually against 60 fertilized *S. ocellatus* eggs and the same number of unfertilized *L. campechanus* and *R. aurorubens* eggs, resulting in positive identification in all cases. By running this LNA Taqman assay on a large number of field-collected eggs, it is possible to quickly and positively estimate field egg abundances, adding a powerful tool for improving ichthyoplankton assessments and determining fine-scale temporal and spatial spawning distributions.

KEY WORDS: Vermilion snapper · Red snapper · Red drum · Lutjanidae · Sciaenidae · Species identification

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INTRODUCTION

Egg production estimates are an important metric in fisheries management models (Hempel 1979, Pepin 2002). However, determining the actual concentrations of eggs in the plankton is often impossible due to the morphological similarity of fish eggs among species. Although morphology has been employed for egg identification (Matarese & Sandknop 1984, Shao et al. 2002, Chen et al. 2007), large variations among few reliable traits, subjective interpretations and long processing times make morphological identification cumbersome and generally unreliable. Instead, a number of molecular DNA techniques have been used with great success, including polymerase chain reaction (PCR) amplification followed by restriction fragment length polymorphism (e.g. Graves et al. 1990, Karaiskou et al. 2005), PCR followed by single strand conformation polymorphism (García-Vázquez et al. 2006), species-specific PCR (Hyde et al. 2005) and DNA sequencing (Paine et al. 2007, Richardson et al. 2007). One of the more promising techniques was developed

*Email: kbayha@ucmerced.edu

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by Taylor et al. (2002), using real-time Taqman PCR probes to identify eggs of 3 species of fish (*Gadus morhua*, *Melanogrammus aeglefinus* and *Merlangius merlangus*). This technique was applied to field-collected eggs from the Irish Sea to illustrate that earlier morphological estimates of cod (*G. morhua*) egg abundances were likely inflated (Fox et al. 2005).

Real-time Taqman PCR uses dual-labeled fluorescent Taqman probe(s) within the traditional methodology of a typical PCR. A Taqman probe is an oligonucleotide that contains both a fluorescent 'reporter' molecule (e.g. FAM, TET, etc.) and a light-absorbing 'quencher' molecule (e.g. black hole quencher, TAMRA, etc.). In short, the quencher inhibits fluorescence when it is in close proximity to a reporter (i.e. when complexed together on the probe). When PCR primers and the Taqman probe anneal during PCR, the probe is hydrolyzed during the extension step. This results in separation of the reporter from the quencher, allowing for fluorescence detection. If the primers and probes are species specific, fluorescence will only occur if DNA from the targeted species is present.

Extremely specific Taqman probes can be designed with a single base pair mismatch preventing probe annealing. However, traditional Taqman probes have been known to be relatively insensitive to single base pair mismatches, so new types of Taqman probes have been developed to increase marker specificity. Two such Taqman probe types are minor groove binding (MGB) and locked nucleic acid (LNA) Taqman probes, which contain alterations that allow for shorter and more specific probes (i.e. single base pair mismatch preventing annealing) as compared to traditional Taqman probes (Kutyavin et al. 2000, Ugozzoli et al. 2004). In general, real-time PCR with Taqman probes tends to be more specific and sensitive than gel-based methods using traditional PCR. With real-time Taqman PCR, the addition of species-specific fluorescent probes adds another level of specificity (above that of only species-specific primers) and DNA amplification detection (via fluorescence detection) is possible with much less initial template DNA.

In the present study, we report the development of a single-tube, single-egg multiplex assay based on the use of 3 species-specific LNA Taqman probes that can be used for identification of field-collected fish eggs. We chose 3 species that represent among the most commercially or recreationally important fishes in the northern Gulf of Mexico, but whose eggs are generally unidentifiable morphologically (Sciaenidae: red drum *Sciaenops ocellatus*; Lutjanidae: red snapper *Lutjanus campechanus* and vermilion snapper *Rhomboplites aurorubens*). By providing the ability to identify individual field-collected fish eggs molecularly, this LNA assay makes it possible to determine the fish egg abundance of these 3 species in the plankton. Such refined measures will provide a better understanding of egg mortality rates and fine-scale spatial and temporal spawning distributions.

MATERIALS AND METHODS

To accomplish our goal of molecular fish egg identification, we developed a routine procedure to apply our multiplex LNA assay on field-collected eggs by modifying the basic 3-step procedure by Fox et al. (2005): (1) rapid extraction of DNA from fish eggs, (2) screening of eggs for positive *Sciaenops ocellatus*, *Lutjanus campechanus* and *Rhomboplites aurorubens* reactions using real-time PCR and the LNA probes and (3) running standard PCR using universal fish primers to ensure that the DNA extracts from any negative LNA tests are amplifiable.

Fish sampling and DNA extraction. Fin clips were collected from adult *Sciaenops ocellatus* (n = 28), *Lutjanus campechanus* (n = 32) and *Rhomboplites aurorubens* (n = 26) originating from various regions in the northern Gulf of Mexico (Table 1). In addition, fin clips were collected from 62 non-targeted fish species in the northern Gulf of Mexico (Appendix 1). For all fin clips, DNA was extracted using the DNAeasy blood and tissue kit (Qiagen).

Choice of genetic region and DNA sequencing. At present, the most common genetic regions deposited in the NCBI (National Center for Biotechnology Information) GenBank for northern Gulf of Mexico fish species are mitochondrial 16S and Cytochrome *b*. Therefore, in order to compare our targeted species' DNA sequences to as many non-targeted local species as possible, we focused on 16S and Cytochrome *b* for the design of species-specific probes. However, there is an ongoing project using a partial sequence from mitochondrial Cytochrome *c* oxidase I (COI) as a fish species marker that has sequenced 4188 species for this

Table 1. Target species tested using species-specific locked nucleic acid (LNA) probes, with nearest locations

Assay and species	Location	n
Red drum probe (FAM)		
Sciaenops ocellatus	Dauphin Island, AL	26
	Gulf Shores, AL	2
Red snapper probe (TET)		
Lutjanus campechanus	Breton Sound, LA	21
	Matagorda Bay, TX	3
	Dauphin Island, AL	8
Vermilion snapper probe (CY5)		
Rhomboplites aurorubens	Dauphin Island, AL	20
	Galveston, TX	6

region to date (www.fishbol.org). So, while we focused on 16S and Cytochrome *b* here, it may be that future projects will utilize COI once a large number of fish COI sequences have been released to GenBank.

Since there is always the possibility of intraspecific polymorphism (within species genetic variation), it is important to avoid these regions when designing species-specific probes that will be effective for all individuals of a species. In order to determine intraspecific polymorphism in the 16S and Cytochrome *b* regions (so as not to place a species-specific probe over a variable region), we sequenced ≥20 ind. from all 3 target species for both 16S and Cytochrome *b*. All primer sequences can be found in Table 2. For all 3 species, 16S was amplified using the primers 16SAR and 16SBR (Palumbi 1996). For *Sciaenops ocellatus*, Cytochrome *b* was amplified using the primers L14725L and HMVZ16 (Santos et al. 2003), while *Lutjanus campechanus* and *Rhomboplites aurorubens* Cytochrome *b* were amplified using the primers CB12F and CB13R (Marko et al. 2004). In all cases, PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 38 cycles of 95°C for 45 s, annealing at 50°C for 1 min and elongation at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The resulting PCR products were then cleaned and sequenced using the original PCR primers (ABI Big Dye Version 3.1) and sequencing products were read using an ABI 3730XL sequencer (University of Washington High-Throughput Genomics Unit, Seattle, WA). Sequencing traces were then assembled using Seqman 5.53 (DNA Star) and corrected by eye. In addition, local *Pogonias chromis* (NCBI Accession No. FJ175391), *Micropogonias undulatus* (NCBI Accession No. FJ-175392), *Bairdiella chrysoura* (NCBI Accession No. FJ175393) and *Leiostomus xanthurus* (NCBI Accession No. FJ175394), not previously deposited in GenBank, were amplified and sequenced for 16S using 16SAR and 16SBR (Palumbi 1996).

Identification of species-specific regions. Intraspecific polymorphic regions were identified by aligning all sequences determined for the present study for each species and those from geographically disparate areas deposited in GenBank against each other using MegAlign 5.53 (DNA Star). In order to identify species-specific regions, their 16S and Cytochrome *b* consensus sequences (with polymorphic regions labeled) were then aligned against GenBank sequences from northern Gulf of Mexico fishes (169 species each for 16S and Cytochrome *b*), as well as the 4 additional local species sequenced for 16S for this project. A potential species-specific region was identified as a region that is highly variable among many species, but not at all variable within the targeted species.

Design and optimization of LNA Taqman probes. All Taqman probes were designed using Beacon Designer 7.01 (Premier Biosoft). LNA probes were designed to span potential species-specific regions (while avoiding all intraspecific polymorphisms), and the discriminatory nature of the LNA probes was

Table 2. Sequences of primers and probes used, including the genetic region targeted and literature source of primers. DMT-174, DMT-180 and DMT-181 are LNA Taqman probes and LNA bases are in capital letters, while normal bases are in small letters. CY5 (CY dye), TET (tetrachloro-6-carboxyfluorescein) and FAM (6-carboxyfluorescin) are 'reporter' dyes, and IBRQ (Iowa Black RQ) and BHQ1 (Black Hole Quencher 1) are 'quencher' molecules. * Primers and probes designed for *Sciaenops ocellatus*; + for *Lutjanus campechanus*; ‡ for *Rhomboplites aurorubens*

	Primer sequence (5′–3′)	Genetic region	Source
Primers			
16SAR	CGCCTGTTTATCAAAAACAT	16S	Palumbi (1996)
16SBR	CCGGTCTGAACTCAGATCACGT	16S	Palumbi (1996)
L14725L	CGAAACTAATGACTTGAAAAACCACCGTTG	Cytochrome *b*	Santos et al. (2003)
HMVZ16	AAATAGGAARTATCAYTCTGGTTTRAT	Cytochrome *b*	Santos et al. (2003)
CB12F	TGGCAAGCCTACGCAAAAC	Cytochrome *b*	Marko et al. (2004)
CB13R	TATTCCGCCGATTCAGGTAA	Cytochrome *b*	Marko et al. (2004)
DMT-187	CAYAAGACGAGAAGACCCT	16S	Present study
DMT-190	TCGAGGTCGTAAACCCCY	16S	Present study
DMT-81*	GGCTTAGCTGTCTCCTTTTTCAA	16S	Present study
DMT-85*	CTGTCTTGGTGTCTAAAGCTCCA	16S	Present study
DMT-172+	GGATTAGCCATCCGTAATTTACA	Cytochrome *b*	Present study
DMT-173‡	GGAGGTTGCGGATTAGTCAG	Cytochrome *b*	Present study
DMT-175+‡	TACAYTACACCTCCGACATCA	Cytochrome *b*	Present study
LNA Taqman probes			
DMT-174‡	CY5-caaTagcTttTTcGtcAgt-IBRQ	Cytochrome *b*	Present study
DMT-180+	TET-tcTcAtcAgtCgccca-BHQ1	Cytochrome *b*	Present study
DMT-181*	FAM-aattCCGACataaGa-BHQ1	16S	Present study

increased by ensuring that the majority of mismatches occurred in the center one-third of the probe. Also, wherever possible, primers were designed to be as specific to the targeted species as possible by placing the 3′ end of the primer on a species-specific base, so as to add a second layer of specificity to the assay. In the end, 3 species-specific LNA probes were designed (Table 2), along with 5 primers (*Lutjanus campechanus* and *Rhomboplites aurorubens* share a forward primer) and were ordered from either Sigma Proligo (DMT-180—*L. campechanus*) or Integrated DNA Technologies (DMT-174—*R. aurorubens* and DMT-181—*Sciaenops ocellatus*). The probes' fluorescent labels (FAM, TET and CY5) were chosen so that they could be differentiated by the Bio-Rad iQ5 system.

All real-time PCR assays were run on a iQ5 real-time thermal cycler (Bio-Rad) using iQ Supermix (Bio-Rad). The multiplex assay was optimized by first testing each primer/probe combination individually with known template DNA at various annealing temperatures, annealing times and primer/probe concentrations. Once optimal conditions were found, a multiplex assay was tested using known template DNA. The final optimal assay consisted of an initial denaturation step at 95°C for 8 min, followed by 38 cycles of 95°C for 15 s and annealing/elongation at 62°C for 35 s, with all probe concentrations at 0.2 µM, Primers DMT-81, DMT-172 and DMT-173 at 0.9 µM and Primers DMT-85 and DMT-175 at 0.4 µM. At the end of each run, the threshold (Ct) values (which show the timing of amplification and indicate initial DNA template concentration) and maximum relative fluorescence units (RFU) were recorded.

Testing of LNA probe specificity. The established LNA assay was then tested against 28 *Sciaenops ocellatus* individuals (Alabama and northwestern Florida coasts), 32 *Lutjanus campechanus* individuals (Texas, Louisiana and Alabama coasts) and 26 *Rhomboplites aurorubens* (Texas and Alabama coasts) individuals in order to ensure that the assay showed a positive reaction to all collected individuals. A positive reaction was determined using the Endpoint program in the Bio-Rad iQ Optical System Software (Version 1.1.1442.0). Secondly, the LNA assay was tested against template DNA from 62 other northern Gulf of Mexico fishes (Appendix 1) so as to ensure that the probes are truly species specific. In order to prove they contained amplifiable DNA, all DNA extracts that showed no reaction to any of the LNA probes were then PCR amplified using the universal fish 16S primers DMT-187 and DMT-190 (Table 2) (see following subsection for design and test of universality).

Determination of appropriate universal fish primer set. As a test to ensure all extracts contained PCR-amplifiable fish DNA (i.e. non PCR-inhibited), we designed a universal fish PCR primer set that could be run in a traditional PCR against extracts showing a negative reaction to all probes. We required that this primer set amplify all fish species collected for the present study, but not human DNA, as there would be a high chance of cross-contamination of human DNA (during handling) that could give a false positive signal. Preliminary attempts to use previously published universal fish primer sets (Kocher et al. 1989, Palumbi 1996, Ivanova et al. 2007) failed to meet the criterion of amplifying DNA from all collected fish species (Table 1, Appendix 1) but not human DNA (from cheek swabs) which could possibly contaminate experimental fish egg extractions. Therefore, we aligned all fish 16S sequences used in the present study and constructed a degenerate primer set that matched all 16S sequences on file, but not human 16S (Table 2). The primer set (DMT-187/190) was tested against all fish species (Table 1, Appendix 1) and human DNA and amplified DNA from all fish, but not human, showing them to be universally applicable fish primers.

Test of LNA assay against known fish eggs. Known eggs of *Sciaenops ocellatus*, *Lutjanus campechanus* and *Rhomboplites aurorubens* were procured either from hatchery spawning (fertilized *S. ocellatus* eggs collected over 2 d from Sea Center Texas, Lake Jackson, Texas) or as unfertilized eggs excised from adult female *L. campechanus* and *R. aurorubens* caught in the vicinity of Dauphin Island, Alabama. For all 3 species, DNA was individually extracted in 96-well plates from a total of 60 eggs. For each, half of these eggs (30) were DNA extracted using the rapid DNAzol Direct (Molecular Research Center) method, in which eggs were burst within the solution and then the solution was heated briefly (95°C for 15 min) and used in the LNA assay. The other eggs were DNA extracted using a modified version of the DNAzol protocol that included a Proteinase K step and a polyacryl carrier. The DNA extracts were then subjected to the multiplex LNA assay using the optimized reagent concentrations and conditions described earlier. All positive reactions were noted, and any DNA extractions that showed a negative reaction were then PCR amplified using our universal primer set to ensure amplifiable fish DNA was present.

RESULTS

DNA sequencing and marker development

For *Sciaenops ocellatus*, 30 ind. were sequenced for 16S (562 base pairs), revealing 7 separate haplotypes (6 variable sites) that differed by 0 to 0.7%. For red drum Cytochrome *b*, 21 ind. were sequenced (724 base

pairs) indicating 10 haplotypes (11 variable sites) that differed by 0 to 0.7%. Twenty-three *Lutjanus campechanus* individuals were sequenced for 16S (561 base pairs), showing no variation in the region (identical to GenBank Accession No.AY857940), while 24 ind. were sequenced for Cytochrome *b* (805 base pairs), revealing 6 haplotypes (8 variable sites) that differed by 0 to 0.6%. Lastly, 24 *Rhomboplites aurorubens* individuals were sequenced for 16S (556 base pairs; all identical to GenBank Accession No. AY857941) and 23 for Cytochrome *b* (749 bp), revealing 2 haplotypes differing by 0.1% (identical to GenBank Accession Nos. AY294200 and AY294198). All novel haplotypes were deposited in NCBI GenBank (NCBI Accession Nos. FJ175395 to FJ175415).

For development of the red drum *Sciaenops ocellatus* probe, 16S proved to be the best region for a species-specific probe, as the designed probe spanned a variable region with an insertion/deletion region, while the variability in Cytochrome *b* made it the best region for *Lutjanus campechanus* and *Rhomboplites aurorubens*. For *S. ocellatus*, the forward primer (DMT-81) was placed over a region relatively specific to *S. ocellatus* (especially at the 3′ end) so that it would anneal to only a few species from GenBank. For *L. campechanus* and *R. aurorubens*, the forward primer (DMT-175) is almost lutjanid-specific, as the 3′ end base matches only 4 non-lutjanid Gulf of Mexico species in GenBank. By decreasing the number of species that could be amplified by the primers, these alterations increased overall discrimination by the probes.

Testing of LNA probe specificity

Testing of the multiplex LNA Taqman assay against DNA from targeted (*Sciaenops ocellatus*, *Lutjanus campechanus* and *Rhomboplites aurorubens*) and 62 non-targeted fish species indicated that the LNA probes designed for the present study are specific to the species for which they were designed. The multiplex LNA assay correctly identified DNA from all 28 *S. ocellatus* individuals, all 32 *L. campechanus* individuals and all 26 *R. aurorubens* individuals. However, the probe failed to react to DNA from 62 non-targeted species commonly found in the northern Gulf of Mexico. Furthermore, PCR amplifications with universal fish primers showed that all 62 DNA extracts contained amplifiable DNA.

Development and testing of a multiplex LNA Taqman protocol for fish egg identification

The multiplex LNA Taqman assay performed perfectly for identifying eggs of the 3 targeted species regardless of the extraction procedure. All 30 *Sciaenops ocellatus* (fertilized), 30 *Lutjanus campechanus* (unfertilized) and 30 *Rhomboplites aurorubens* (unfertilized) eggs extracted using the rapid DNAzol Direct method showed positive detection for the appropriate LNA probe, with the same number of eggs extracted using the traditional DNAzol method (with Proteinase K and a polyacryl carrier) showing the same pattern. However, in all cases, egg DNA extracted using the traditional DNAzol method showed lower Ct values and higher final RFU values (Table 3). In addition, a higher proportion of eggs extracted using DNAzol Direct amplified extremely late in the assay (with Ct values >30 cycles), as compared to those extracted using traditional DNAzol (3 for *S. ocellatus*, 3 for *L. campechanus* and 1 for *R. aurorubens*).

DISCUSSION

The multiplex LNA Taqman real-time PCR assay developed in the present study proved to rapidly and accurately identify DNA extracted from eggs of the red drum *Sciaenops ocellatus*, red snapper *Lutjanus campechanus* and vermilion snapper *Rhomboplites aurorubens*. The species-specific nature of the probes was ensured by designing them using DNA sequences from 25 or more specimens of known identity and GenBank sequences from 169 Gulf of Mexico species. As such, the technique perfectly identified DNA from all

Table 3. Results of tests of LNA Taqman assay on known eggs of 3 target species for 2 DNA extraction techniques, including average threshold values (Ct ± SD) and maximum relative fluorescent units (RFU ± SD) (n = 30). Since values were collected over multiple real-time PCR runs, Ct values are not comparable in a quantitative sense, but meant for a general comparison of DNA extraction techniques

Taxon	DNAzol Direct		Traditional DNAzol	
	Ct	Maximum RFU	Ct	Maximum RFU
Sciaenops oscillatus	24.65 ± 4.90	1579.80 ± 549.30	19.510 ± 4.00	3982.53 ± 787.8
Lutjanus campechanus	25.95 ± 3.42	876.30 ± 210.10	19.60 ± 3.42	2080.60 ± 463.20
Rhomboplites aurorubens	26.44 ± 1.87	730.60 ± 181.36	19.74 ± 2.02	2520.50 ± 293.20

known samples of the 3 species and showed null reactions for all 62 non-target northern Gulf of Mexico species. The application of this assay to individual field-collected fish eggs in a high throughput 96-well plate method can produce quick and accurate estimations of fish eggs in the wild, greatly increasing our understanding of the early life histories of these ecologically and economically important species, improving stock assessments for fisheries management and determining specific spawning times and geographic regions.

Given the large biodiversity of fish species in the northern Gulf of Mexico, there was little expectation that we could have accounted for or acquired all possible fish DNA sequence information during the probe design process. Therefore, we designed the most specific type of Taqman probe available to us, utilizing the recently developed LNA Taqman probes. For their fish egg assay, Taylor et al. (2002) and Fox et al. (2005) used MGB probes. These probes are a variation on the traditional Taqman probe, and incorporate an MGB moiety that increases stability and specificity by artificially increasing the probe melting temperature. Ultimately, this allows for short (13 to 20 base pairs long) and more specific probes (Kutyavin et al. 2000). However, the cross-platform functionality of these probes is problematic, since they use proprietary fluorescent dyes (Applied Biosystems) beyond FAM, TET and HEX, which we could not differentiate on the Bio-Rad iQ5.

We developed a procedure based on the more broadly functional LNA Taqman probes. LNA Taqman probes are similar to traditional Taqman probes except that they incorporate LNA bases, nucleic acid analogs containing a modified ribose moiety that increases the affinity for matching nucleic acids, effectively increasing the probe-melting temperature and allowing for shorter and more specific probes (Ugozzoli et al. 2004). However, unlike MGB probes, we were able to create a multiplex assay using >2 probes that could be differentiated with a non–Applied Biosystems, real-time PCR thermal cycler. By using LNA Taqman probes, we were able to further increase the species specificity of our multiplex assay.

In order to detect all 3 species in a single tube, we designed our species-specific LNA probes to incorporate fluorescent molecules with excitation wavelengths that could be differentiated on the Bio-Rad iQ5 (FAM, TET and CY5). However, the abilities of the machine allow for 2 more species-specific probes to be added to the assay (labeled with TEX-613 and CY3), bringing the total number of species that could be detected to 5. Although multiplex assays with this many different probes can prove problematic when run against multiple DNA templates (due to competition between templates for PCR reactants), since this assay is run on a single DNA template (i.e. single egg), such problems would be minimal.

Fish egg DNA extraction methods

Numerous rapid DNA extraction methods have been developed for small metazoans (Schizas et al. 1997, Leutbecher 2000), including those specifically designed for extracting DNA from fish eggs (Aranishi 2005). For our study, we wanted a method that could be done rapidly on a single 96-well plate and could be used on the relatively small eggs from the present study (~700 to 1000 µm). The Aljanabi & Martinez (1997) method requires 2 plates, and the Aranishi (2005) method, while successful on larger eggs in that study (>1 mm), was largely unsuccessful in our study. Therefore, we pursued 2 DNAzol (Molecular Research Center) methods (traditional DNAzol and DNAzol Direct), since neither required the transfer of samples to a second 96-well plate. The traditional DNAzol method required alcohol precipitation, but all could be done on a single plate. The DNAzol Direct method entails simply bursting the eggs in a stabilizing liquid, followed by heating, a very short centrifuge step (<1 min) and immediate progress to the PCR steps, making it the faster of the 2 methods. However, runs of DNA released via the short DNAzol Direct protocol were more likely to give late Ct values, indicative of very low concentrations of PCR-available template. Recently, we have experimented with including a short Proteinase K digestion (<3 h), followed by dissolution in DNAzol Direct, and all indications are that the addition of that step produces data similar to those found with the traditional DNAzol method. Given that this method is rapid and not very labor intensive, the DNAzol Direct method shows great promise in reducing DNA extraction/release times for high-throughput analyses.

Usefulness of the LNA assay for understanding the early life history of fishes

Egg identification based on morphology is generally impossible for our 3 targeted species (red drum, red snapper and vermillion snapper), so a rapid molecular technique for species identification will be extremely useful. Field-collected red drum eggs are morphologically similar to those of other sciaenids, and can be positively identified only by incubating the eggs until hatching and then identifying the larvae (Holt et al. 1988). Egg descriptions are available for 5 species of western Atlantic snappers, including red snapper, but unavailable for the remaining 13 species, including vermilion snapper (Lindeman et al. 2006). However, the morphological characters delineating the 5 snapper egg descriptions overlap to such an extent that their usefulness is questionable.

Positive identification of field-collected red drum, red snapper and vermilion snapper eggs from surveys can provide valuable fisheries-independent data for fisheries managers. Spawning locations can be inferred from the spatial distribution of eggs, along with knowledge of the local current regime. Likewise, data from egg surveys can be valuable in species stock assessments. Such are invaluable for other species, such as the Pacific sardine, for which stock assessments rely on spawning stock biomass estimates provided by fish egg surveys (Lo & Macewicz 2006). Stock assessments for Atlantic mackerel and other scombrids are also fine-tuned with data collected from egg surveys (Stratoudakis et al. 2006). Similarly, the identification of early larval forms is also beneficial and provides an additional tool for resource managers. Variability in the recruitment of fishes to the adult population is largely related to the variability encountered in vital rates during the larval stages (Cowan & Shaw 2002). Previously unknown mortality rates for the early larval stages can now be estimated, allowing for more robust larval fish indices for use in assessing spawning stock biomass and predicting fluctuations in recruitment (Pepin 2002). Such indices have recently been used in stock assessments for several economically important species in the northern Gulf of Mexico, including Atlantic bluefin tuna (Scott et al. 1993) and king mackerel (Gledhill & Lyczkowski-Shultz 2000), and have been investigated for use in assessments of red snapper (SEDAR 2005) and vermilion snapper (SEDAR 2006).

In addition to strict management applications, comparing early life histories between species can provide information on the ecology of marine fishes and their population dynamics (Miller 2002). Vertical distribution patterns of eggs and larval fishes, for example, can have implications for advective transport to nursery areas or juvenile habitats (Govoni & Pietrafesa 1994, Hare et al. 1999). Interestingly, adult red snapper and vermilion snapper occupy the same habitat (low-profile hard bottom), but the pelagic juveniles display different settlement patterns in that red snapper settle onto more open, low-relief relic shell beds (Szedlmayer & Conti 1999) and vermilion snapper settle onto the reef habitat itself, often schooling above it (SEDAR 2006). Little is known about the spawning locations of either species. Quantifying such differences in the sources and fate of fish eggs and larvae could yield additional insights into the early life-history strategies between similar species.

Given the general morphological similarity of fish eggs in the plankton, future studies of field egg abundance will ultimately require rapid, high-throughput techniques for identifying eggs through non-morphological means. Here, we have designed a molecular technique to identify fish eggs rapidly and in large numbers, allowing for accurate estimations of fish egg abundance for 3 species of Gulf of Mexico fishes. Furthermore, we have modified earlier methods to reduce time taken and avoid the use of labor-intensive DNA extraction methods, and have employed Taqman probes that are more broadly functional across multiple real-time PCR platforms. By allowing direct estimation of fish egg abundance in the Gulf of Mexico, this LNA Taqman assay will generate more accurate ichthyoplankton assessments, providing resource managers with valuable fisheries-independent estimates of spawning biomass, as well as information on spatial and temporal spawning distributions for these commercially and recreationally important fish.

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Appendix 1. Non-target species tested using species-specific LNA probes. All species were collected from the vicinity of Dauphin Island, Alabama, USA. *Larval fishes

Acanthocybium solandri
Archosargus probatocephalus
Arius felis
Bagre marinus
Bairdiella chrysoura
Balistes capriscus
Brevoortia patronus
Caranx crysos
Caranx hippos
Centropristis ocyurus
Centropristis philadelphica
Chaetodipterus faber
_Chloroscombrus chrysurus_*
Coryphaena hippurus
Cynoscion arenarius
Cynoscion nebulosus
_Cynoscion nothus_*
Diplectrum sp.*
Echeneis naucrates
Elops saurus
Epinephelus morio
_Etropus crossotus_*
Eucinostomus gula
Euthynnus alletteratus
Fistularia tabacaria
Lagodon rhomboides
Larimus fasciatus
Lobotes surinamensis
Lutjanus synagris
Megalops atlanticus
Menticirrhus americanus
Menticirrhus littoralis
Micropogonias undulatus
Mycteroperca microlepis
Opsanus beta
Orthopristis chrysoptera
Pagonias cromis
Paralichthys albigutta
Paralichthys lethostigma
_Peprilus alepidotus_*
Pomatomus saltatrix
Priacanthus arenatus
Prionotus scitulus
Pristipomoides aquilonaris
Rachycentron canadum
Scomberomorus cavalla
Scomberomorus maculatus
Selene vomer
Saurida brasiliensis
Seriola dumerili
Serraniculus pumilio
Sphoeroides nephelus
Sphyraena guachancho
Leiostomus xanthurus
Sphyraena barracuda
Stenotomus caprinus
Syacium papillosum
Symphurus sp.*
Thunnus albacares
Thunnus atlanticus
Trachinotus carolinus
Trichiurus lepturus

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