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Genetic Tools to Identify Species of *Seriola* in the U.S. South Atlantic and Gulf of Mexico

Mark A. Renshaw
Texas A&M University

John R. Gold
Texas A&M University

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SHORT PAPERS AND NOTES

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GENETIC TOOLS TO IDENTIFY SPECIES OF *SERIOLA* IN THE U.S. SOUTH ATLANTIC AND GULF OF MEXICO.—Four species in the carangid genus *Seriola* (greater amberjack, *Seriola dumerili*; lesser amberjack, *Seriola fasciata*; banded rudderfish, *Seriola zonata*; and Almaco jack, *Seriola rivoliana*) support commercial and recreational fisheries in the U.S. South Atlantic Ocean (hereafter Atlantic) and the northern Gulf of Mexico (hereafter Gulf). Of these, greater amberjack is by far the most preferred, with commercial landings in the Atlantic and Gulf in 2008 being over four-fold greater than landings of the other three species combined (http://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html). Assessments of greater amberjack resources in the Gulf (Turner et al., 2000; SEDAR9, 2006), have indicated that the stock was overfished and that overfishing is still occurring. A major concern (RFSAP, 1996) regarding estimates of greater amberjack fishing mortality is misidentification by both commercial and recreational fishers of undersized greater amberjack as one of the other three species, especially banded rudderfish (E. Matheson, pers. comm.) All four species are similar in appearance, with identification of species based on characters such as length of anal fin base, length of second dorsal fin lobe, shape of vomer, and gill raker and fin-ray counts (Berry and Burch, 1979; Manooch and Raver, 1984; Shipp, 1986). The misidentification issue with respect to greater amberjack is underreporting of greater amberjack landings and possibly underestimates of fishing mortality. What is needed is a simple, reliable method to identify unequivocally each species of *Seriola* regardless of fish size and morphological character overlap.

Direct sequencing of mitochondrial DNA (mtDNA) has been used to identify individual species from closely related congeners in a number of exploited fishes, including sharks (Pank et al., 2001; Abercrombie et al., 2005), salmonids (Purcell et al., 2004), and sciaenids (Anderson et al., 2009). Typically, the mtDNA sequences employed display relatively large interspecies differences but relatively small intraspecies differences (Greig et al., 2005). A second approach (Purcell et al., 2004) has been to use restriction-fragment-length polymorphisms (RFLPs), where one amplifies an mtDNA fragment via polymerase chain reaction (PCR), then digests the fragment with one or more restriction endonucleases that provide diagnostic banding patterns. A third approach is to identify a suite of PCR priming sites that also generate diagnostic banding patterns (Pank et al., 2001; Abercrombie et al., 2005) and can be multiplexed (Shivji et al., 2002) to significantly reduce time and/or expense relative to both direct sequencing and RFLP analysis. In this note, we report the design and implementation of multiplexed PCR primers for mtDNA sequences that unequivocally allow identification of each of the four species of *Seriola*.

Materials and methods.—A total of 84 fish were used in the study: 28 putative greater amberjack (all from John's Island, SC), 32 putative Almaco jack (13 from John's Island, SC; 19 from the Florida Keys), 17 putative banded rudderfish (six from John's Island, SC; 11 from Panama City, FL), and seven putative lesser amberjack (all from John's Island, SC). Fin clips were taken from individual fish and placed in 95% ethanol (fish sampled in waters off of Florida) or sarkosyl-urea (fish sampled in waters off of South Carolina). Identification as to species was made in the field by colleagues who provided the samples (see Acknowledgments). Fixed samples were transported to our laboratory at Texas A&M and stored at room temperature. Genomic DNA was extracted from each sample using a standard phenol-chloroform protocol (Sambrook et al., 1989).

PCR primers L13562 and H14718 (Inoue et al., 2000) were used to amplify a fragment containing sequences of the mitochondrial protein-coding genes ND-5 and ND-6. The 50-µl PCR mixture comprised the following: 0.5 µm of each primer, 1× PCR buffer (5× colorless Go*Taq*® Flexi buffer, Promega), 2 mM $MgCl_2$, 200 µM dNTPs, 2.5 U Go*Taq*® Flexi DNA polymerase (Promega), and 5 µl of DNA (unknown concentration). The PCR protocol was an initial denaturation at 95°C for 3 min; 45 cycles of denaturation at 95°C for 30 sec, annealing at 54°C for 45 sec, elongation at 72°C for 2 min; and a final elongation at 72°C for 20 min. PCR products were electrophoresed on a 2% agarose gel; successful amplifications were band-cut and cleaned with QIAquick gel extraction kits (Qiagen). Fragments were sequenced in both directions, using the L13562/H14718 primers and ABI BigDye Terminator version 1.1; products

were electrophoresed on an ABI 3100 automated DNA sequencer (Applied Biosystems) and sequences were edited with Sequencher 3.0 (Gene Codes). Sequences on the ends were trimmed to remove regions that were difficult to score; the remaining 1,113-bp fragment was compared across all 84 sampled individuals.

An mtDNA haplotype data file was generated in DnaSP (Rozas et al., 2003) in order to compare haplotypes within and among species. The Kimura two-parameter model (Kimura, 1980), as employed in MEGA4 (Tamura et al., 2007), was used to generate estimates of average evolutionary distance within and between species. MEGA4 also was used to generate a neighbor-joining (NJ) tree; bootstrap values for nodes in the inferred NJ topology were generated from 500 replications. Initial evaluation of the NJ topology revealed four individuals that had been likely misidentified in the field; three individuals identified as lesser amberjacks had mtDNA haplotypes virtually identical (≤0.8% sequence divergence) to those of 31 of the individuals identified as Almaco jacks, whereas one individual identified as an Almaco jack had a haplotype virtually identical (≤0.4% sequence divergence) to those of 28 individuals identified as greater amberjacks. The degree of difference between the average evolutionary distance within each species compared to that between species (see Results) essentially eliminated the possibility that these four individuals were correctly identified. The four individuals were then reassigned to the correct species and the average evolutionary distances and the NJ topology re-estimated.

Consensus mtDNA sequences for each species were generated and aligned using ClustalX 2.0.11 (Larkin et al., 2007). Species-specific PCR primers were then designed based on regions where a sequence of bases that were unique to each species was identified. Each species-specific primer site was located in a different region of the 1,113-bp fragment such that different (species-specific) size fragments would be generated when each species-specific primer was used in tandem with the H14718 primer. Species-specific primers designed were as follows: greater amberjack, *Sdu*-L (5′-CCAAG TATACGACCATATAAGTGA-3′); lesser amberjack, *Sfa*-L (5′-CCGCCTCTAATCTTCCTTT-3′); banded rudderfish, *Szo*-L (5′-CGCTAATAAC TAGCATTCACC-3′); and almaco jack, *Sri*-L (5′-GCTAGCTGCCCTGACAGTC-3′). All four species-specific primers were combined with the H14718 primer in a single 10-µl PCR multiplexed mix that included the same recipe of reagents previously mentioned but with a few differences: 0.2 µm of each primer, 0.5 U Go*Taq*® Flexi DNA polymerase (Promega), and 1 µl of DNA (unknown concentration). The PCR protocol was the same as mentioned previously except with a decrease in the number of cycles (38 total). The H14718/L13562 primer pair also was evaluated in the PCR multiplexes as a positive control for PCR amplifications (Shivji et al., 2002). This primer pair yielded a second distinguishable band that was larger than all four species-specific bands. PCR amplifications with species-specific primers pairs and both with and without the H14718/L13562 primer pair were performed for all 84 individuals; fragments generated were size-separated on 2% agarose gels, stained with ethidium bromide, and viewed under ultraviolet (UV) light.

Results and discussion.—The distribution of mtDNA haplotypes is given in Table 1; there were a total of 39 haplotypes, none of which were shared between species. The number of haplotypes within each species ranged from three (*S. fasciata*, four individuals) to 14 (*S. zonata*, 17 individuals). The mean evolutionary distances (sequence divergence), given in Table 2, were two orders of magnitude higher between species than within species; within-species values ranged from 0.1% (*S. fasciata*) to 0.8% (*S. rivoliana*), whereas between-species values ranged from 11.4% (*S. dumerili* vs *S. rivoliana*) to 17.8% (*S. rivoliana* vs *S. zonata*). The NJ topology (Fig. 1) presents a visual representation of the high levels of between-species difference as compared to within-species difference. Bootstrap values uniting mtDNA haplotypes within each species were 100%.

Multiplex PCR amplifications, using the species-specific PCR primers, were successful for all 84 individuals. A subset of the individuals assayed is shown in Figure 2. Species-specific bands (fragments) were *S. dumerili*, ~300 bp; *S. zonata*, ~500 bp; *S. fasciata*, ~700 bp; and *S. zonata*, ~950 bp). All species-specific bands were easily distinguishable by size on a 2% agarose gel stained with ethidium bromide and viewed under UV light. Addition of the L13562 primer successfully added a positive control band (~1100 bp) that was distinguishable by size from the four species-specific bands (Fig. 2). In a number of instances, there was noticeably weaker amplification of the positive control (Fig. 2: lanes 14, 16, and 25); Shivji et al. (2002) observed similar results in their study of pelagic sharks. In general, smaller products are amplified more efficiently during PCR, providing a plausible explanation for weaker amplification of the larger positive-control band described both in Shivji et al. (2002) and the present study.

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TABLE 1. Summary of mtDNA haplotypes for 84 individuals, obtained from a 1,113-bp fragment of the protein coding ND-5 and ND-6 mitochondrial genes. MtDNA haplotype sequences are available online under their corresponding GenBank accession numbers. Sampling localities, South Carolina (SC) and Florida (FL), are noted with the number of individuals sharing the same mtDNA haplotype within each locality in parentheses.

Haplotype	GenBank	*Seriola* species	Location (sample size)
1	GU014709	Greater amberjack (*S. dumerili*)	SC (2)
2	GU014710	Greater amberjack (*S. dumerili*)	SC (5)
3	GU014711	Greater amberjack (*S. dumerili*)	SC (3)
4	GU014712	Greater amberjack (*S. dumerili*)	SC (1)
5	GU014713	Greater amberjack (*S. dumerili*)	SC (2)
6	GU014714	Greater amberjack (*S. dumerili*)	SC (2)
7	GU014715	Greater amberjack (*S. dumerili*)	SC (1)
8	GU014716	Greater amberjack (*S. dumerili*)	SC (1)
9	GU014717	Greater amberjack (*S. dumerili*)	SC (1)
10	GU014718	Greater amberjack (*S. dumerili*)	SC (3)
11	GU014719	Greater amberjack (*S. dumerili*)	SC (5)
12	GU014720	Greater amberjack (*S. dumerili*)	SC (3)
13	GU014721	Lesser amberjack (*S. fasciata*)	SC (2)
14	GU014722	Lesser amberjack (*S. fasciata*)	SC (1)
15	GU014723	Lesser amberjack (*S. fasciata*)	SC (1)
16	GU014724	Almaco jack (*S. rivoliana*)	SC (4), FL (2)
17	GU014725	Almaco jack (*S. rivoliana*)	SC (5), FL (7)
18	GU014726	Almaco jack (*S. rivoliana*)	SC (1)
19	GU014727	Almaco jack (*S. rivoliana*)	SC (1)
20	GU014728	Almaco jack (*S. rivoliana*)	SC (1)
21	GU014729	Almaco jack (*S. rivoliana*)	SC (1), FL (8)
22	GU014730	Almaco jack (*S. rivoliana*)	SC (1)
23	GU014731	Almaco jack (*S. rivoliana*)	SC (1)
24	GU014732	Almaco jack (*S. rivoliana*)	SC (1)
25	GU014733	Almaco jack (*S. rivoliana*)	FL (1)
26	GU014734	Banded rudderfish (*S. zonata*)	FL (1)
27	GU014735	Banded rudderfish (*S. zonata*)	FL (1)
28	GU014736	Banded rudderfish (*S. zonata*)	SC (1), FL (1)
29	GU014737	Banded rudderfish (*S. zonata*)	FL (1)
30	GU014738	Banded rudderfish (*S. zonata*)	FL (1)
31	GU014739	Banded rudderfish (*S. zonata*)	FL (1)
32	GU014740	Banded rudderfish (*S. zonata*)	FL (1)
33	GU014741	Banded rudderfish (*S. zonata*)	FL (2)
34	GU014742	Banded rudderfish (*S. zonata*)	FL (1)
35	GU014743	Banded rudderfish (*S. zonata*)	FL (1)
36	GU014744	Banded rudderfish (*S. zonata*)	SC (2)
37	GU014745	Banded rudderfish (*S. zonata*)	SC (1)
38	GU014746	Banded rudderfish (*S. zonata*)	SC (1)
39	GU014747	Banded rudderfish (*S. zonata*)	SC (1)

TABLE 2. Kimura two-parameter mean evolutionary distances: sequence divergence within (diagonal) and between (below diagonal) species.

	Greater amberjack	Lesser amberjack	Almaco jack	Banded rudderfish
Greater amberjack	0.004			
Lesser amberjack	0.130	0.001		
Almaco jack	0.114	0.144	0.008	
Banded rudderfish	0.158	0.157	0.178	0.004

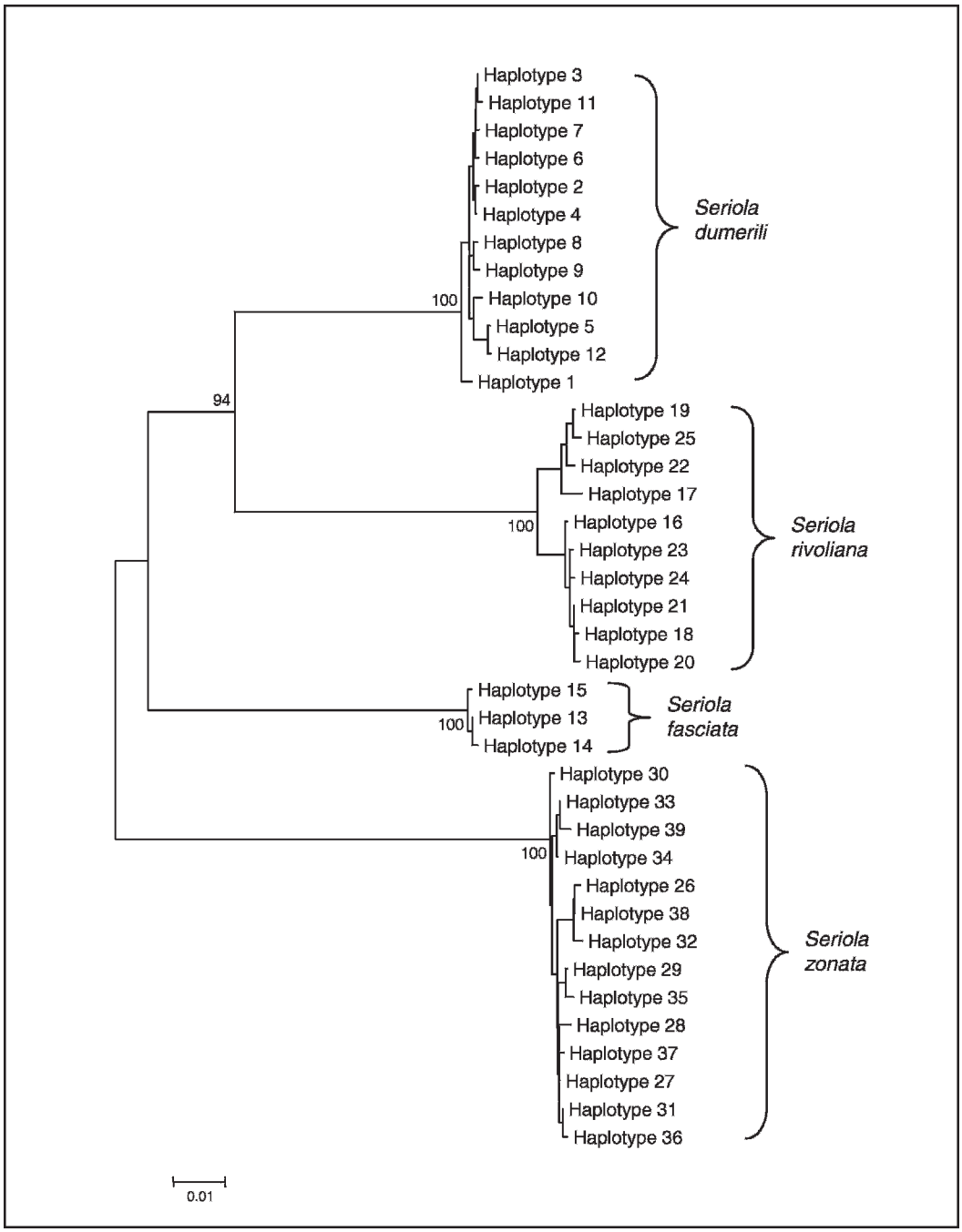

Fig. 1. Neighbor-joining tree derived from a 1,113-bp fragment of the protein coding ND-5 and ND-6 mitochondrial genes. Sequences are clustered into four species groups: greater amberjack (*Seriola dumerili*), lesser amberjack (*Seriola fasciata*), Almaco jack (*Seriola rivoliana*), and banded rudderfish (*Seriola zonata*). Node bootstrap values were generated from 500 replications. Scale bar represents 1% sequence divergence.

The weaker amplification of the positive-control sequence did not detract from the ability to identify each species of *Seriola* as the intensity of the species-specific bands was unaffected.

The four species of *Seriola* in the Atlantic and Gulf are difficult to identify correctly, in part because of overall similarity in appearance, and in part because characters used to identify each

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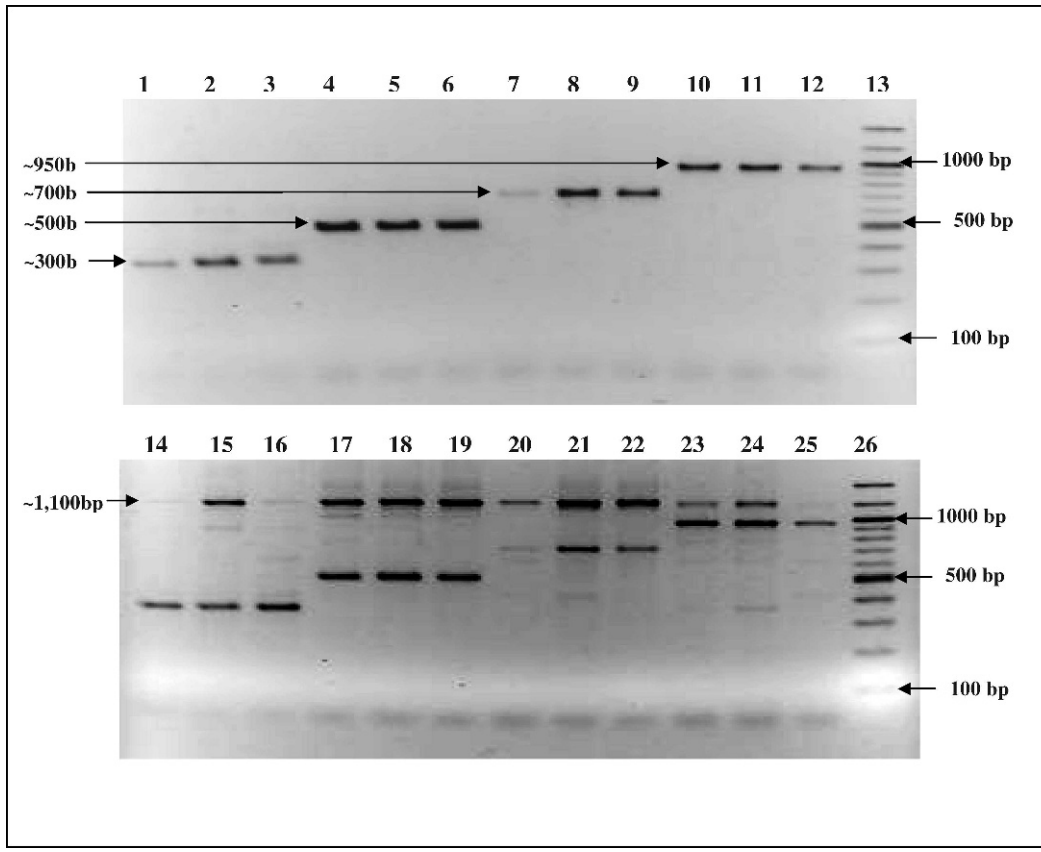

Fig. 2. Sample gel image of species-specific fragments (bands). Lanes 1–12 are without the positive control; lanes 14–25 include the positive control (~1,100-bp fragment). Lanes 1–3/14–16 are greater amberjack (*Seriola dumerili*, ~300 bp); lanes 4–6/17–19 are banded rudderfish (*Seriola zonata*, ~500 bp); lanes 7–9/20–22 are lesser amberjack (*Seriola fasciatus*, ~700 bp); and lanes 10–12/23–25 are Almaco jack (*Seriola rivoliana*, ~950 bp). Lanes 13 and 26 are sizing ladders (100-bp DNA ladder, New England BioLabs), with 100-, 500-, and 1,000-bp fragments marked on the right. The same three individuals from each species were assayed in lanes 1–12 and 14–25.

species (length of anal fin base and second dorsal fin lobe, shape of vomer, and gill raker and fin-ray counts) often have overlapping ranges (Berry and Burch 1979; Manooch and Raver, 1984; Shipp, 1986). This difficulty was evident in our study as four of the individuals assayed were misidentified in the field. The two–order-of-magnitude difference in sequence divergence of the ND-5/ND-6 fragment between as compared to within the four species of *Seriola*, however, indicates that the mtDNA fragment is a reliable tool to identify each species unequivocally.

Application of this method on a larger scale is necessary to estimate the overall magnitude of misidentification in landings of species of *Seriola*. Ideally, landings of species of *Seriola* in the commercial catch could be sampled and the percentage of misidentification recorded. This ostensibly would allow for correction of landings relative to individual species that could be incorporated into estimates of fishing mortality, especially for greater amberjack. A caveat is that the geographic distributions of the four species, in particular the circumglobally distributed greater amberjack and Almaco jack (Shipp, 1986), make it possible that mDNA haplotypes have yet to be sampled and that these additional haplotypes may contain mutations in priming sites that would inhibit amplification of the species-specific fragments developed in this study. More comprehensive sampling could address this concern; adjustments to the species-specific primers in response to additional data, however, should be straightforward. In addition, sequencing of the ND-5/ND-6 fragment undoubtedly would provide unequivocal identification of each species.

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Mark A. Renshaw and John R. Gold, *Center for Biosystematics and Biodiversity, Texas A&M University, College Station, TX 77843-2258, USA.*

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