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ScienceDirect
Progress in Natural Science 18 (2008) 1233–1238
Progress in Natural Science
www.elsevier.com/locate/pnsc

Sequencing and analysis of the complete mitochondrial DNA of Russell's snapper (*L. russellii*)

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Received 24 March 2008; received in revised form 24 April 2008; accepted 25 April 2008

Abstract

The entire mitochondrial DNA sequence (mitogenome) of Russell's snapper (*Lutjanus russellii*) was determined using long PCR and primer-walking methodology, representing the first complete mitogenome accessioned for Lutjanid fishes (16,505 bp, GenBank Accession No. EF514208). The mitogenome was similar in gene composition and order to those of other vertebrates, having 37 structural genes, i.e., two ribosomal RNAs, 22 transfer RNAs, and 13 protein-coding genes. Phylogenetic analyses based on the mtDNA sequence of Russell's snapper supported a close relationship between Lutjaninae and Caesioninae, consistent with taxonomic hypotheses based on morphology. More studies utilizing mitogenomes are needed to resolve high-level relationships among snappers.

Keywords: *Lutjanus russellii*; Mitochondrial DNA; Mitogenome; Complete mtDNA sequence; Long PCR

1. Introduction

Fishes of the genus *Lutjanus* Bloch (Perciformes: Lutjanidae) are distributed over coral reef and hard-bottom habitats throughout the tropical and subtropical oceans worldwide. In the South China Sea (SCS), this genus is composed of approximately 20 indigenous species that serve as an economically important food resource for developing countries in this region. For the most part, phylogenetic relationships among SCS snappers are poorly resolved, principally because taxa are morphologically similar. However, restriction fragment length polymorphism (RFLP) assays of amplified mitochondrial DNA (mtDNA) genes, e.g., cytochrome *b* (cyt *b*) and cytochrome *c* oxidase subunit I, have proven to be useful in resolving some of the phylogenetic relationships among morphologically similar snappers [1–5]. Technical issues, such as identifying informative restriction enzymes and improving resolution for small fragments, still need be resolved for high-level taxonomic classifications.

Because nucleotide variation in mtDNA represents an informative source of phylogenetic data, more mitochondrial genomic (mitogenomic) information is needed. Recent advances in DNA technology have made mtDNA sequencing cheaper and quicker, which in turn has made complete mitogenomes easier to obtain. To date, 1898 mitogenomes for 1283 species are accessioned in GenBank, including those of many fish species. For example, Nagase et al. [6] determined the mitogenome of the Japanese flying fish (*Cypselurus hiraii*); and Hurst et al. [7] sequenced the mitogenome of the Atlantic salmon *Salmo salar*.

In this study, we determined the complete mitogenome of the commercially important Russell's snapper (*Lutjanus russellii*) using an approach that combined long PCR and primer walking. Primer design was based on sequence data from two mitochondrial genes, cyt *b* and cytochrome *c*

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1002-0071/$ - see front matter
doi:10.1016/j.pnsc.2008.07.001

oxidase subunit II (COII), obtained in prior studies of Russell's snapper [5]. The resulting mitogenomic data provided key information not only for further population studies of this snapper species, but also for phylogenetic studies of the genus *Lutjanus*.

2. Materials and methods

2.1. Specimen and tissue sample

The specimen of *L. russellii* was caught offshore in the SCS (Zhanjiang, China). Its organs were immediately frozen in liquid nitrogen, and were stored at −70 °C.

2.2. Preparation of DNA

Total DNA was prepared from the specimen's muscle tissue by proteinase K digestion according to conventional procedures [8].

2.3. Mitochondrial DNA purification by long PCR

Two long PCR primers (Left1-COII/Left1-cyt *b* and Right1-cyt *b*/Right1-COII) designed with reference to the complete nucleotide sequences of cyt *b* and COII, respectively, were used to amplify the entire mitochondrial genome.

Long PCR assays were conducted with a Hema 8000 thermal cycler in a 50 μl reaction containing 5 μl 10× PCR buffer, 5 μl dNTPs (2 mM), 3 μl $MgCl_2$ (25 mM), 2.5 μl each primer (4 μM), 2.5 U of LA *Taq* DNA (Takara), and approximately 100 ng template DNA. The PCR profile was as follows: 94 °C for 80 s; 30 cycles of 94 °C for 42 s, 55 °C for 30 s, 68 °C for 8 min; and 72 °C for 10 min. PCR products were electrophoresed on a 0.7% agarose gel, and subsequently stained with ethidium bromide for band characterization via ultra violet transillumination.

2.4. Segment PCR and sequencing

Primers for the overlapping PCR fragments, designed with reference to the complete mitochondrial genome sequences from *Pterocaesio tile* (GenBank Accession No. AP004447), are listed in Table 1.

Segment PCR was accomplished using long PCR products (diluted 1:20) as templates. The reaction profile was as follows: 94 °C for 80 s; 30 cycles of 94 °C for 42 s, 47–55 °C (depending on primer annealing temperature) for 30 s, 68 °C for 100 s; and 72 °C for 10 min. PCR products were electrophoresed and characterized as described above. PCR products were ligated into the pUCm-T vector (Bio Basic Inc.), and were transformed into DH5α competent cells of *Escherichia coli*. The presence of recombinant DNA was confirmed by PCR amplification using M13 universal primers. Positive clones were sequenced on an Applied Biosystems 377 automated DNA sequencer from both directions by primer walking using standard M13 sequencing primers and other sequencing primers (Table 1).

Table 1
PCR and sequencing primers used in the analysis of the Russell's snapper mitogenome

Primers	Primer sequences (5′–3′)	T_m (°C)	Length (bp)
PCR primers			
COII-F	CAAGCCAACCACATAACC	53	866
COII-R	TCGGGAGTCACCAGTCTTTA		
cyt *b*-F	GTGACTTGAAAA ACCACC GTTG	50	1343
cyt *b*-R	CTCCATCTCCGGTTTACA AGAC		
Left1-COII	CCAGACAGCCTTTATCGC	55	7189
Left1-cyt *b*	CGAAGAAGCGAGTGAGG		
Right1-cyt *b*	TCGCCTCAATCCTCGTTC	55	8536
Right1-COII	CCATAGCCACAATAATGTAAAG		
LRU1-COII-F	ATGCCCATTGTAGTTGAAGC	50	1937
LRU1-ND3-R	AGGTGTTATTTGCGGTAGTC		
LRU2-ND3-F	CATCCTCGCAATCGTCTC	53	2029
LRU2-tRNA-Leu-R	TCAAATTAGCTCGGGTTT		
LRU3-ND4-F	CGAGCTAATTTGAGGTTGA	47	2682
LRU3-cyt *b*-R	GCTTGCCATTTAGAGGTTC		
LRU4-cyt *b*-F	TCCTGGCTTCTATTCTCGT	47	1283
LRU4-12S rRNA-R	AGCTTTCTAGGGCTCATCT		
LRU5-tRNA-Phe-F	GTTAAGATGAGCCCTAGAAA	53	2621
LRU5-16S rRNA-R	ATAGATAGAAACTGACCTGGAT		
LRU6-16S rRNA-F	CCGCTATTAAGGGTTCGTT	53	2739
LRU6-tRNA-Tyr-R	TAGATGGATGCTCGCTGGT		
LRU7-tRNA-Asn-F	CGAGCATCCATCTACCTTT	50	2036
LRU7-COII-R	GAGACCATCGCCACAATAA		
Sequencing primers			
LRU1.3P	GCTTCTAACATCCCTCATAC		
LRU2-2.4P	TTTATTGCCTTATCCCTATG		
LRU2-2.6P	CTTGTCGCTCTCCTCCTTCT		
LRU3.6P	CATAGCCTCAACGACGAACA		
LRU3.4P	TAGTCACAGCCAACAACATA		
LRU3.REV1	ACCAACGGATGAGCTGTTAT		
LRU3.REV2	AAGCGCCGCTGAATAAGCAA		
LRU5.3P	AGACAAGTGATTGCGCTACC		
LRU5.4P	GGACTTGGCGGTGCTTTAGA		
LRU6.3P	TTGATATTGGTTGCCTTGTT		
LRU6.4P	CCGTAATTGACCTGAACTTG		

2.5. Mitogenome analysis

BLASTN [9] programs were used to search nucleotide databases using sequenced nucleotide segments. In MEGA 4.0 [10], homologous sequences from GenBank were used to aid alignment and to identify certain codon positions and frame-shifts. Then, all fragments were assembled with DNASTAR (http://www.dnastar.com/), and virtual enzyme digestions were conducted for the complete

mitochondrial DNA sequence with pDRAW32 (revision 1.1.94, http://www.acaclone.com). Using sequences identified in the BLASTN searches, the phylogenetic relationships were analyzed by the neighbor-joining (NJ) method online. In MEGA, all 13 protein-coding regions were automatically translated, and their amino acid frequencies were computed. We used TRNAscan-SE 1.23 (http://lowelab.ucsc.edu/tRNAscan-SE), an online program for the improved detection of transfer RNA (tRNA) genes, to detect all tRNAs and to construct clover-leaf structures. Finally, the program Sequin (http://www.ncbi.nlm.nih.gov/Sequin/index.html) was used for mitogenome submission.

3. Results

3.1. Mitochondrial DNA amplification by PCR

Two pairs of long PCR primers were used to amplify Left1 (7 kb) and Right1 (8.5 kb). Seven primer pairs (Table 1) were used to amplify contiguous, overlapping segments of Left1 and Right1 (Fig. 1). The complete sequence of *L. russellii* assembled with cyt *b*, COII, and seven segments in Left1 or Right1 was 16,505 bp. All sequence data are available from GenBank under the Accession No. EF514208.

3.2. Comparison of restriction fragment length polymorphisms (RFLPs)

Xiao et al. [11] isolated the mtDNAs from livers of Russell's snapper, and conducted a restriction fragment length polymorphism (RFLP) analysis using 19 restriction endonucleases. Here, using pDRAW32, we 'digested' virtually our mtDNA sequence with the same 19 restriction endonucleases. Our restriction-fragment profiles were nearly identical to those reported by Xiao et al. (Table 2).

3.3. BLASTN of mitogenomes

The mitochondrial genomic sequence in *L. russellii* was similar to other vertebrate mitochondrial genomes with

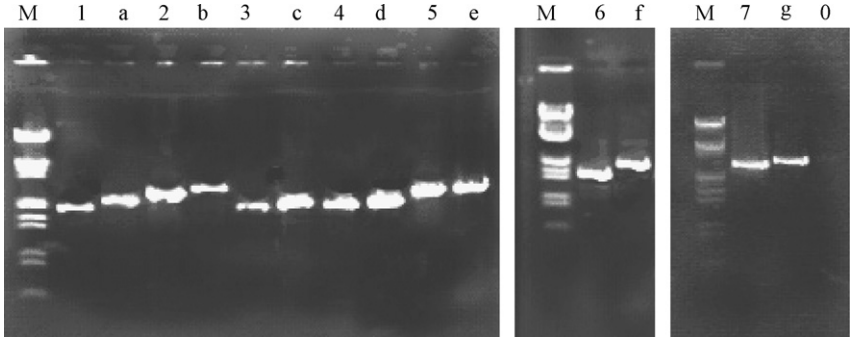

Fig. 1. PCR amplification products and corresponding recombinant clones. M, λ DNA/HindIII markers; 0, negative control; 1–7, the PCR products of primer pairs LRU2, LRU5, LRU1, LRU7, LRU3, LRU4 and LRU6, respectively: a–g, corresponding recombinant clones of PCR products.

Table 2
Numbers and sizes of mtDNA restriction fragments for actual and virtual single-enzyme digestions

	Real enzyme digestion			Virtual enzyme digestion		
Enzyme	No.	Size (kb)	Sum (kb)	No.	Size (bp)	Sum (bp)
BamHI	2	13.28; 4.16	17.44	2	12,045; 4460	16,505
BglI	1	17.37	17.37	1	16,505	16,505
BglII	1	17.37	17.37	1	16,505	16,505
DraI	5	3.75; 3.47; 2.53; 2.43; 2.03	17.21	9	3913; 3589; 2701; 2561; 2159; 768; 419; 283; 112	16,505
EcoRI	0			0		
EcoRV	1	17.37	17.37	2	16,469; 36	16,505
HindIII	3	10.68; 4.16; 3.01	17.85	4	8689; 4118; 3075; 623	16,505
HinfI	n	n		32		16,505
HpaI	4	7.02; 4.94; 4.16; 1.41	17.53	4	6928; 4838; 3330; 1409	16,505
KpnI	0			0		
MluI	0			0		
PstI	1	17.37	17.37	2	15,811; 694	16,505
PvuII	0			2	16,472; 33	16,505
SalI	0			0		
ScaI	5	6.56; 3.65; 3.16; 2.88; 1.50	17.75	5	5951; 3580; 3165; 2291; 1518	16,505
SmaI	1	17.37	17.37	1	16,505	16,505
StyI	n	n		23		16,505
XbaI	3	11.14; 4.28; 2.16	17.58	3	9785; 4486; 2234	16,505
XhoI	1	17.72	17.72	1	16,505	16,505

Note: The fragments not detected completely are marked with an "n".

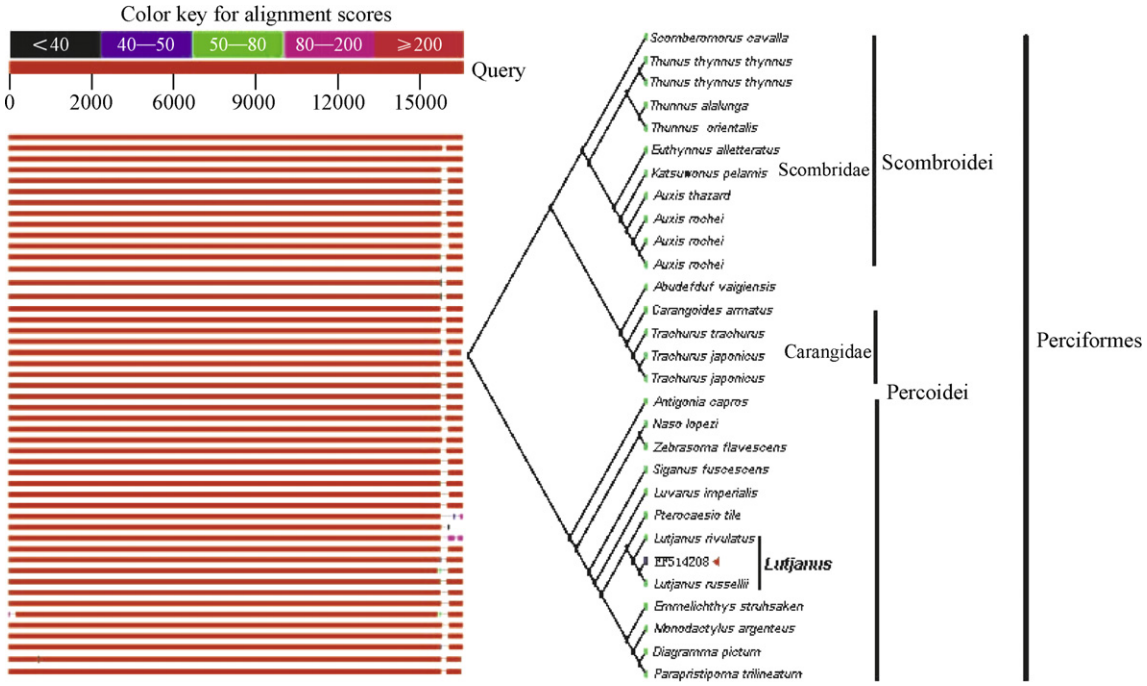

Fig. 2. The result of BLASTN of Russell's snapper mitochondrial DNA.

respect to gene order and genomic organization. Phylogenetic relationships derived from mtDNA sequences of *L. russellii*, *Lutjanus rivulatus*, and other ichthyic counterparts (Fig. 2) were congruent with those derived from morphological taxonomic analyses. As expected, high homogeneities were observed between *L. russellii* and *L. rivulatus*, as well as between members of Lutjaninae and Caesioninae (Caesioninae: *Pterocaesio tile*).

3.4. Mitogenome content and base composition

As in the other bony fishes [12], the genome content of *L. russellii* included two rRNA, 22 tRNA, 13 protein-coding genes, and a control region. Most genes were encoded on the H-strand, except for the ND6 and eight tRNA genes (Pro, Glu, Ser, Tyr, Cys, Asn, Ala and Gln). Excluding the control region, non-coding sequences were composed of 59 nucleotides, including a 27 bp long fragment and 8 shorter fragments that isolated genes.

3.4.1. Protein-coding genes

Among the 13 protein-coding genes of *L. russellii*, there were two reading-frame overlaps on the same strand (ATPases 8 and 6 shared 10 nucleotides; ND4L and ND4 shared seven nucleotides). As in other bony fishes, all the mitochondrial protein-coding genes began with an ATG start codon, except for COI, which started with GTG. Open reading-frames ended with a partial stop codon T for ND4 and cyt *b*, or with TAG for COI, II, and ND3; the remainder ended with TAA. An anti-G bias was observed at third codon positions (6.5%). Pyrimidines were over-represented in the second codon positions, as has been noted for other vertebrate mitochondrial genomes, owing to the hydrophobic character of the proteins. The average usages of amino acid for 13 proteins were Cys (C) 2.2, Ser (S) 4.6, Asp (D) 5.3, Arg (R) 5.9, Lys (K) 6.9, Glu (E) 7.5, Tyr (Y) 7.9, Gln (Q) 8.6, Trp (W) 9.0, His (H) 9.5, Asn (N)10.3, Met (M) 12.0, Val (V) 15.1, Gly (G) 16.7, Phe (F) 17.0, Pro (P) 18.5, Ile (I) 20.4, Thr (T) 23.9, Ala (A) 25.4 and Leu (L) 69.7.

3.4.2. tRNA and ribosomal RNA genes

The mitochondrial genome contained 22 tRNA genes, including 18 single style genes ($tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Asp}$, $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Glu}$, $tRNA^{Thr}$ and $tRNA^{Pro}$) and two pairs of double style genes ($tRNA^{Leu}$ and $tRNA^{Ser}$).

The tRNA genes ranged in size from 65 to 75 nucleotides, which allowed the encoded tRNA to fold into the characteristic clover-leaf secondary structure. All postulated clover-leaf structures contained 7 bp in the amino acid stem, 5 bp in the TψC stem, 5 bp in the anticodon stem, and 4 bp in the DHU stem. The G–U wobble and other atypical pairings were in the stem regions of $tRNA^{Phe}$, $tRNA^{Pro}$, $tRNA^{Glu}$ and $tRNA^{Ser\ (UGA)}$.

The 12S and 16S rRNA genes of *L. russellii* were 954 and 1681 bp in length, respectively.

3.4.3. Control region (D-loop)

As in most vertebrates, the control region of *L. russellii* was located between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes. This region (837 bp in length) appeared to contain both conserved sequence blocks (CSBs) and high variational blocks

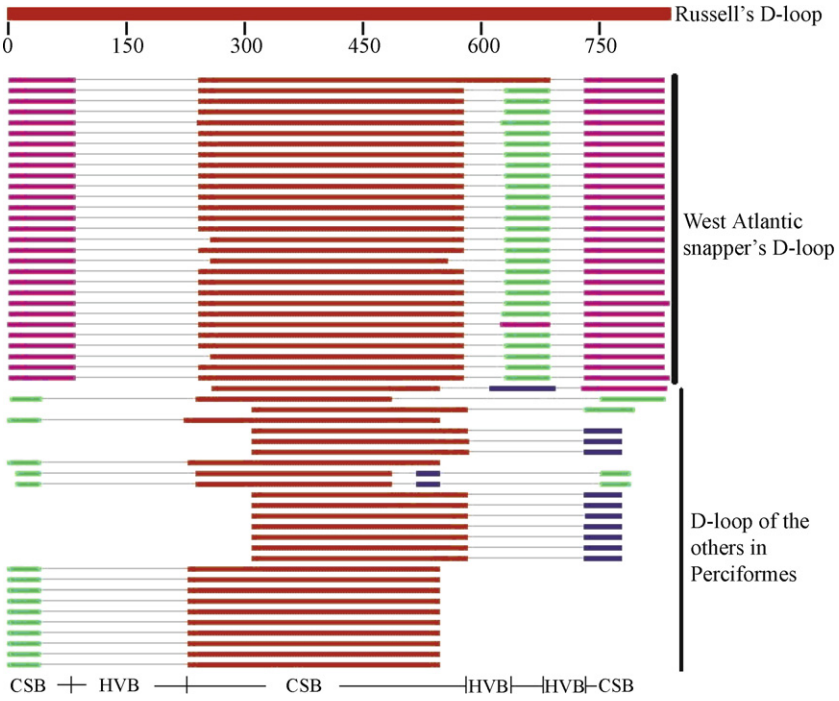

Fig. 3. The BLASTN result of D-loop. CSB, conserved sequence block; HVB, high variational block.

(HVBs) (Fig. 3), also characteristic of vertebrate control regions.

4. Discussion

We used a long PCR technique to produce templates for the subsequent primer-walking PCR. Two sets of species-specific primers were designed with reference to the complete nucleotide sequences of cyt *b* and COII [5]. The lengths of the two long PCR products were 7 kb and 8.5 kb, respectively, nearly the entire mitogenome of Russell's snapper. This technique was successfully used to obtain adequate amounts of mitochondrial DNA from even a small specimen.

Using purified mtDNA or amplified fragments of mtDNA of Russell's snapper for population analysis via RFLPs was both inexpensive and quick. However, the shortcomings of these methods (lack of information regarding restriction enzyme sites, and difficulties in purifying mtDNA and resolving small fragments) are apparent (Table 2). In this study, the analysis of virtual enzyme digestion not only confirmed the results of mtDNA-RFLP by Xiao et al. [11], but also provided further information regarding the choice of restriction enzymes. Moreover, the long PCR technique described herein could produce longer fragments for PCR-RFLP.

The HVB regions depicted in Figs. 2 and 3 will likely be very useful in further studies of rapidly speciating vertebrates. For example, Lockhart et al. [13] resolved the phylogeny of swordtail fishes using the D-loop sequence.

The perciform family Caesionidae (Fusiliers) is closely related to the snapper family (Lutjanidae). However, it possesses several adaptations for a planktivorous mode of life, such as its elongated fusiform body, small mouth, and deeply forked caudal fin. During the day, fusiliers occur in large zooplankton-feeding schools in mid-water over coral reefs, along steep outer-reef slopes, and around deep lagoon pinnacles. Although they are active swimmers, they often pause to pick zooplankton or occupy cleaning stations. At night, they take shelter within the reef. Fusiliers are important food fishes, and are also used as bait in tuna fisheries [14].

In Fishbase (http://fishbase.fishinfo.cn/search.php), the subfamily Caesioninae belongs to the family Caesionidae, and the family Lutjanidae is divided into four subfamilies (Etelinae, Lutjaninae, Paradicichthyinae, Apsilinae). However, Miller et al. [15] called the taxonomic position of the Caesioninae into question. Based on 16S ribosomal RNA and cytochrome *b* mitochondrial DNA sequence, they posited that the fusiliers should be nested within the subfamily Lutjaninae, making Caesionidae a synonym of Lutjanidae. In our comparison of mitogenomic data (Fig. 2), a close relationship between Lutjaninae and Caesioninae was observed. Further taxonomic sampling and mitogenomic sequencing efforts may clarify the phylogenetic relationships among snappers.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No. 30671610). We thank Dr. Michael Tringali at the Florida Fish and Wildlife Commission for the revision of this manuscript.

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