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UNIVERSAL PRIMERS FOR AMPLIFICATION OF THE COMPLETE MITOCHONDRIAL CONTROL REGION IN MARINE FISH SPECIES

© 2012 Y.-Z. Cheng, T.-J. Xu*, X.-X. Jin, D. Tang, T. Wei, Y.-Y. Sun, F.-Q. Meng, G. Shi, R.-X. Wang**

Key Laboratory for Marine Living Resources and Molecular Engineering, College of Marine Science, Zhejiang Ocean University, P.R. China

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Through multiple alignment analysis of mitochondrial tRNA-Thr and tRNA-Phe sequences from 161 fishes, new universal primers specially targeting the entire mitochondrial control region were designed. This new primer set successfully amplified the expected PCR products from various kinds of marine fish species, belonging to various families, and the amplified segments were confirmed to be the control region by sequencing. These primers provide a useful tool to study the control region diversity in economically important fish species, the possible mechanism of control region evolution, and the functions of the conserved motifs in the control region.

***Keywords*: mitochondrial DNA, control region, universal primers, marine fish species.**

The decline of fisheries around the world has created much interest in using genetic marker tools to help establish conservation policies that can protect the local adapted fish stocks by regulating fishing activities [1, 2]. Mitochondrial DNA has been widely used as a marker for evolutionary and population studies because of its compact size, nearly complete maternal inheritance, and fast evolutionary rate [3, 4]. Many studies of mitochondrial DNA has focused on the major noncoding region control region or D-loop, located between the $tRNA^{Thr}$ and $tRNA^{Phe}$ genes, due to its complex structure and supposedly fast evolutionary rate. Reduced function constrains make some portions of the control region (such as the first hypervariable region, HVR-1) evolve much faster than the average mitochondrial DNA sequences [5], thus, such portions are expected to be a more sensitive tool for detecting population subdivisions. Alternatively, absence of conserved blocks in the control region is often observed in some taxa [6, 7], and these large variations imply the rapid evolution of the structure of the control region, which may help mining the information on the structure-function relationships in the control region.

Despite the value of this non-coding region, its use has been somewhat limited by the requirement for rapid and large-scale amplification and sequencing. Designing universal primers to amplify the entire control region can facilitate obtaining the target sequences. Previous attempts of designing universal mitochondrial control region primers have met some success [8, 9], however, because of the differences in the methods for designing the universal primers (e.g. the differences in the used template gene and whether the primers contain the degenerate bases), the amplification scope of the available primer sets is relatively limited. These situations require designing more universal control region primer sets based on a different conserved gene template, and combining with the existing primers to rapidly and simply amplify as many fish mitochondrial control regions as possible.

The length and nucleotide sequence of $tRNA^{Thr}$ and $tRNA^{Phe}$ can be highly conserved between considerably distant taxa. On this basis, we choose these genes as the template. The DNA sequence data of the $tRNA^{Thr}$ and $tRNA^{Phe}$ of 161 fishes [10] from GenBank was aligned to determine the conserved sequence regions. These conserved sequence regions were then compared with the GenBank resources by BLAST to insure their conservation across different fish species. One set of primers was designed from these conserved sequence regions. Primer information is shown in Table 1.

To confirm the usability and robustness of the universal primers, we performed PCR on DNA from various samples (table 2). DNA was extracted from the muscle of 45 fish samples using the standard phenol-

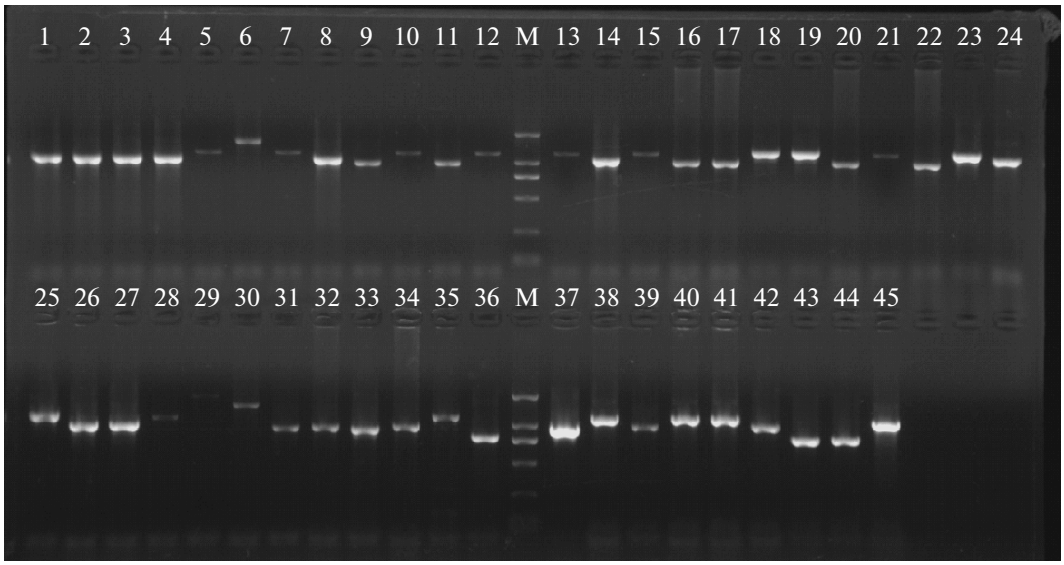
* E-mail: tianjunxu@163.com; **wangrixin1123@126.com

Table 1. Primers used in this study

Primer name	Sequence
Marinefish-Dloop-Thr-F	AGCACCGGTCTTGTAAACCG
Marine-Dloop-Phe-R	GGGCTCATCTTAACATCTTCA

chloroform procedure [11]. The PCR mixture consisted of 0.2 μM of each primer, 5.0 μL of 10× *Taq* Plus polymerase buffer, 0.2 mM dNTPs, 2 units of *Taq* Plus DNA polymerase with proof-reading characteristic ("TIANGEN"), and 1 μL of the DNA template. PCR was performed on a BIO-RAD S1000. The conditions of the PCR were as follows: predenaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1.5 min; and final extension at 72°C for 5 min. The PCR products were electrophoresed on a 2% agarose gel ("Biogel") in TBE buffer (50 mM Tris, 1 mM EDTA, and 48.5 mM boric acid) and purified using the QIAquick PCR purification kit ("Qiagen"). The purified PCR products were sequenced using an ABI 3130 genetic analyzer.

In all cases the obtained products of a single band were of the expected size (Figure). The sequences of the amplified products were compared with the known sequences in GenBank and thus confirmed the products to be the control region. Broad reactivity primers are very beneficial for amplification of DNA from an unknown region [12] and it is evident that regions residing in the $tRNA^{Thr}$ and $tRNA^{Phe}$ loci in the mitochondrial genome among fishes are more strictly conserved than the regions in the 12S rRNA and *Cyt b* loci. Functionally important regions usually show the strict sequence conservation. Using these highly conserved regions, we have successfully developed universal primers for amplifying a relatively wide variety of fish mitochondrial control regions and facilitating future research.



Images of the PCR amplicons for the 45 fish samples.

Table 2. Species used in this study

Number	Name	Family
1	*Sciaenops ocellatus*	Sciaenidae
2	*Ilisha elongata*	Clupeidae
3	*Carassius auratus*	Cyprinidae
4	*Barbuligobius boehlkei*	Gobiidae
5	*Acanthogobius hasta*	Gobiidae
6	*Boleophthalmus pectinirostris*	Periophthalmidae
7	*Acanthogobius ommaturus-1*	Gobiidae
8	*Acanthogobius flavimanus*	Gobiidae
9	*Scartelaos viridis*	Periophthalmidae
10	*Acanthogobius hasta*	Gobiidae
11	*Tridentiger bifasciatus*	Gobiidae
12	*Priacanthus macracanthus*	Priacanthidae
13	*Scomber australasicus-1*	Scombridae
14	*Mugil cephalus*	Mugilidae
15	*Cociella crocodilus*	Platycephalidae
16	*Larimichthys polyactis*	Sciaenidae
17	*Hexagrammos otakii*	Hexagrammidae
18	*Carangoides oblongus*	Carangidae
19	*Nibea japonica*	Sciaenidae
20	*Chrysochir aureus*	Sciaenidae
21	*Trichiurus japonicus*	Trichiuridae
22	*Argyrosomus argentatus*	Sciaenidae
23	*Psenopsis anomala*	Centrolophidae
24	*Siniperca chuatsi*	Serranidae
25	*Scomber australasicus-2*	Scombridae
26	*Branchiostegus japonicus*	Branchiostegidae
27	*Pleuronichthys cornutus*	Pleuronectidae
28	*Konosirus punctatus-1*	Clupeidae
29	*Sebastes marmoratus*	Scorpaenidae
30	*Coilia mystus*	Engraulidae
31	*Miichthys miiuy*	Sciaenidae
32	*Collichthys lucidus*	Sciaenidae
33	*Collichthys niveatus*	Sciaenidae
34	*Odontamblyopus lacepedii*	Gobiidae
35	*Pagrosomus major*	Sparidae
36	*Hapalogenys mucronatus*	Pomadasyidae
37	*Hapalogenys nitens*	Pomadasyidae
38	*Oplegnathus fasciatus*	Oplegnathidae
39	*Parargyrops edita*	Sparidae
40	*Acanthogobius ommaturus-2*	Gobiidae
41	*Nibea albiflora*	Sciaenidae
42	*Acanthopagrus lat*	Sparidae
43	*Sardinella aurita*	Clupeidae
44	*Konosirus punctatus-2*	Clupeidae
45	*Acanthopagrus schlegelii*	Sparidae

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