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DNA Amplification and Nucleotide Sequence Determination of a Region of Mitochondrial DNA in the Sea Snake, *Laticauda Semifasciata*

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We determined the nucleotide sequence of a region of the 12S ribosomal RNA (rRNA) gene in the mitochondrial DNA (mtDNA) of the sea snake, *Laticauda semifasciata*, using the polymerase chain reaction (PCR). We synthesized oligonucleotide primers according to the nucleotide sequence of human mt DNA 12S rRNA gene and found that the target sequence (386bp) of the sea snake mtDNA could be amplified with these primers. The nucleotide sequence of the amplified region of the sea snake mt DNA was determined on six separate plasmid clones for each individual snake DNA and matched completely among the DNA samples of three sea snakes. The sequence homology in the region of the mtDNA 12S rRNA gene between *L. semifasciata* and human is 69.1%.

Introduction

Mitochondrial DNA (mtDNA) is a circular double-stranded exonuclear DNA and is approximately 16.5kbp long. It encodes 16S and 12S ribosomal RNA (rRNA), 22 transfer RNA and 13 enzymes which are required in oxidative phosphorylation. Since the first determination of the complete nucleotide sequence of the human mtDNA by ANDERSON, *et al*,[1] the nucleotide sequence data of mtDNA have been accumulated from a wide variety of species. MtDNA is inherited maternally, does not recombine,[2] and its base mutation rate is 5 to 10 times faster than that of nuclear DNA.[3] Knowledge of the mtDNA sequences in many species has been providing new insights into our understanding on the genetic divergence of species.[4,5,6] We applied the polymerase chain reaction (PCR) to the DNA of the sea snake, *Laticauda semifasciata*, using human mtDNA specific primers and determined the nucleotide sequence of the 12S rRNA coding region of the mtDNA.

Materials and Methods

I Preparation of DNA samples

Approximately 0.5g of liver tissue from each sea snake was transfered to a sterile 15ml centrifuge tube, avoiding contaminations. 7ml of the digestion solution consisting of 10mM Tris-HCl (pH 8.0), 2mM EDTA, 10mM NaCl, 1% SDS, 1% dithiothreitol and proteinase K (0.5mg/ml)[7] was added and mixed gently at 37 ℃ for 10 to 15 hours to solubilize the tissue. An equal volume of saturated phenol was added to the solution and mixed with gentle shaking at room temperature for several hours. The mixture solution was centrifuged at 3000g for 15 minutes and the water phase was trasfered to a new tube. The phenol extraction was repeated until the water phase became colorless. Finally, the water phase was extracted once with chloroform/isoamylalcohol (24:1) and the DNA was precipitated by addition of 0.1 volume of 5M NaCl and 2.2 volume of ethanol. The DNA was dissolved in 10mM Tris-HCl (pH 8.0) and 1mM EDTA and the concentration was adjusted to 0.5mg/ml.

II Polymerase chain reaction

A pair of oligonucleotide primers, H1478 (5'TGACTGCAGAGGGGTGACGGGGCGGTGTGT-3') and L1091 (5'-AAAAAGCTTCAAACTGGGATTAGATACCCACTAT-3') were synthesized, using a DNA synthesizer, Cyclone™ Plus (Milligen/Bioresearch Division, Millipore). The sequence of these primers was chosen from the 12S rRNA coding region of the human mtDNA[1,4] (Fig. 1) and the expected size of the amplified DNA is 450 bp including 64 bp of the primer sequences. The PCR mixture was composed of 0.5 μg of the template DNA, 2mM of dNTP, 50 picomole of each primer and 2 units of Tth polymerase of Thermus thermophilus HB8 (Toyobo) in 100μl of the reaction buffer (20mM Tris-HCl, pH8.5, 50mM KCl, 0.05% Tween 20, 2mM MgCl2, 0.01% gelatin and 0.5% NP40).[8] Routinely, thirty cycles of PCR were carried out in Minicycler™ (MJ Research) and the following is the protocol for amplification conditions.

Program I

Denaturation at 94 ℃	for 2 minutes: in the first cycle for 1 minute: in the second to 30th cycle
Annealing at 52 ℃	for 1 minute
Extension at 72 ℃	for 1 minute: in the first to 29th cycle

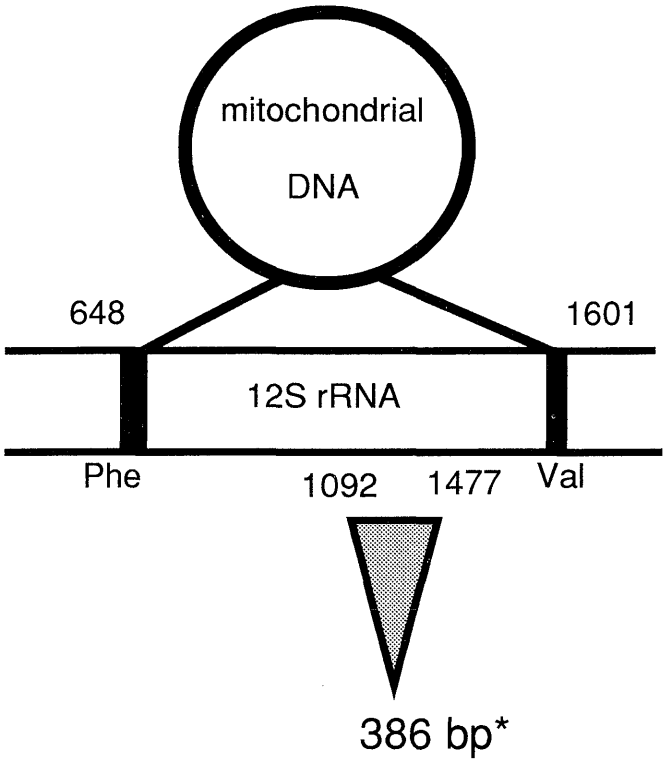

Fig. 1. Localization of the target sequence of the polymerase chain reaction (PCR) in the mitochondrial DNA (mtDNA).
*The primer sequences are not included in the estimated size of the amplified DNA.

	for 6 minutes: in the final cycle
Program II	
Denaturation at 94 ℃	for 2 minutes: in the first cycle
	for 1 minute: in the second to 30th cycle
Annealing at 54 ℃	for 0.5 minute
Extension at 72 ℃	for 1 minute: in the first to 29th cycle
	for 6 minutes: in the final cycle

Amplification conditions and the number of reaction cycles were varied from a DNA sample to another in order to optimize the quality and the yield of the PCR products. A portion of the PCR product was electrophoresed in a 0.6% agarose gel, stained with ethidium bromide and detected under UV light (Fig. 2).

III Sequence determination of the amplified DNA

A. Purification of the amplified DNA
The amplified DNA fragment was further purified for cloning and sequence determination. The following three procedures were experimented.
1) Ethanol precipitation
After PCR, the reaction mixture was extracted once with chloroform. The DNA was precipitated by addition of 0.1 volume of 3M ammonium acetate and 2.2 volume of ethanol, recovered by centrifugation at 15000 rpm for 10 minutes and used directly for the fill-in reaction with E. coli DNA polymerase I Klenow fragment and kination with T4 polynucleotide kinase.
2) The second PCR on the amplified DNA
The original PCR product was electrophoresed on a 0.6% agarose gel. A gel piece containing the 450 bp DNA fragment was cut out, frozen in a microfuge tube and crushed with a pipette tip. After centrifugation at 12000 rpm for 10 minutes, the DNA was recovered in the supernatant. A portion (1 μl) of the DNA solution was subjected to the second PCR, using the same pair of primers. The annealing step in the second PCR was performed at 60 to 62 ℃ for 30 seconds. A single DNA band of 450 bp was eluted from a gel, extracted once with phenol/chloroform and used for further experiments.
3) Purification of the amplified DNA by a high performance liquid chromatography (HPLC)
A portion of the PCR product was electrophoresed through a 0.6% agarose gel and when a single DNA band of 450 bp was recognized on a gel, the remaining portion of the PCR product was applied on a Waters Gen-Pak DNA column. Elution was made with a linear gradient of 0.05 to 0.45M NaCl in Tris-HCl (pH 8.0) and 1mM EDTA over 30 minutes.
4) Nucleotide sequence determination of the amplified DNA

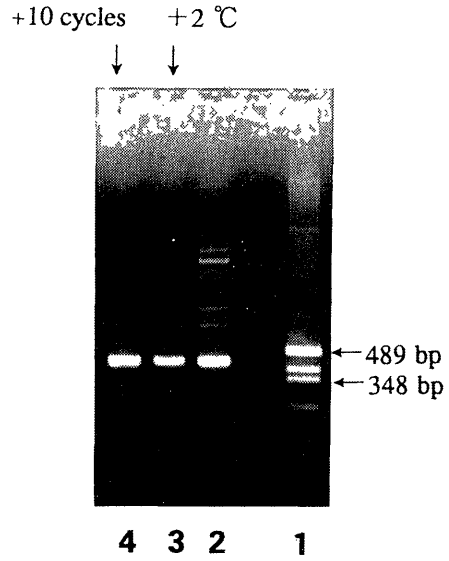

Fig. 2. Agarose gel electrophoresis of the PCR product from L. semifasciata DNA.
Lane 1 DNA size marker
Lane 2 The PCR product of the sea snake DNA by annealing at 52 ℃
Lane 3 The PCR product of the sea snake DNA by annealing at 54 ℃
Lane 4 The PCR product after 40 cycles of amplification (annealing at 54 ℃)

After purification of the amplified DNA by one of the above methods, the DNA was bluntended with Klenow fragment of *E.coli* DNA polymerace I and phosphorylated with T4 polynucleotide kinase.[9] The phosphorylated DNA was then ligated into the *Hinc*II site of pUC118 DNA using a DNA ligation kit (Takara). The recombinant plasmids were transfected into DH5α competent cells prepared by a cold culture method.[12] A single colony containing the recombinant plasmid was picked out from an agar plate containing X-gal, Isopropyl β-D-Thiogalactopyranoside and ampicillin. The plasmid DNA was purified by a modified method of BIRNBOIM and DOLY[11] and the nucleotide sequence was determined by the SANGER method,[12] using Sequenase™ Version 2.0 (Toyobo) and [α -^{35}S] ATP.

Results

Analysis of the amplified products

The DNA amplified from the 12S rRNA coding region of *L. semifasciata* mtDNA was analyzed by electrophoresis in a 0.6% agarose gel and a major DNA band of approximately 450 bp was recognized (Fig. 1). Although the amplification conditions were varied for each DNA sample, the annealing at 54 ℃ generally produced the more homogeneous DNA fragment of 450 bp than at 52 ℃ (Fig. 1, lane 2 and 3). When the PCR cycles were increased from 30 to 40, both the quality and the quantity of the PCR product appeared to be improved (Fig. 1, lane 4). Elongation of the extension time helped to increase the yield of the amplified DNA, however some extra DNA bands were observed. The doubled PCR was rarely required although it was certainly a useful method prior to the nucleotide sequence determination of the PCR product.

The nucleotide sequence of the amplified region of the sea snake mtDNA

The nucleotide sequence of the portion of the 12S rRNA gene in *L. semifasciata* mtDNA was determined on six recombinant clones containing the DNA produced by a single PCR for each individual sea snake DNA sample. Fig.

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                10         20         30         40         50         60         70         80
MLS1S1  GCCTAGCCAT AACCAAACAG CCTAATAACC AGCTGTTCGC CAAATAACTA CGAGTTATAA CTTAAAATTT AAAAGACTTG
MLS1S2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S3  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S4  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S6  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                90         100        110        120        130        140        150        160
MLS1S1  ACGGTACTTC ACACCGACCT AGAGGAGCCT GTCTAATAAC CGATAACCCA CGATTAACCC AACCTGTTCT AGCCCAATCA
MLS1S2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S3  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S4  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S5  ---------- ---------- ---------- ---------- ----C----- ---------- ---------- ----------
MLS1S6  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                170        180        190        200        210        220        230        240
MLS1S1  GTCTATATAC CGCCGTCGCC AGCTTACCTT ATGAAAGAAA CAAAGTAAGC CTAATAATAA CACATTAACA CGACAGGTCG
MLS1S2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S3  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S4  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S6  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                250        260        270        280        290        300        310        320
MLS1S1  AGGTGTAACT AATGAACAGG ACCAAGATGG GCTACACTTT CTAACCTAGA AAAAACGAAC AGACTATGAA ACTAGAAACT
MLS1S2  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S3  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S4  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S5  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
MLS1S6  ---------- ---------- ---------- ---------- ---G------ ---------- ---------- ----------

                330        340        350        360        370        380
MLS1S1  GAAGGCGGAT TTAGCAGTAA GATGGGAACA AACACACCCA ACCGAAGATA ACGCAATGAA GTGTGC
MLS1S2  ---------- ---------- ---------- ---------- ---------- ---------- ------
MLS1S3  ---------- ---------- ---------- ---------- ---------- ---------- ------
MLS1S4  ---------- ---------- ---------- ---------- ---------- ---------- ------
MLS1S5  ---------- ---------- ---------- -G-------- ---------- ----G----- ------
MLS1S6  ---------- ---------- ---------- ---------- ---------- ---------- ------
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Fig. 3. Nucleotide sequence of the region of the 12S rRNA gene in L. semifasciata mtDNA.
MLS1S1 to MLS1S6 represent the sequences determined on six plasmid clones containing the amplified DNA (MLS1) of a sea snake DNA sample (LS1).

3 shows the nucleotide sequence obtained on these six plasmid clones. The size of amplified DNA was 386 bp excluding the primer sequence, which matches with the size of the corresponding region in the human mtDNA. A typical example of the radioautograph for sequence analysis is presented in Fig. 4 to show the reproducibility of the experiment.

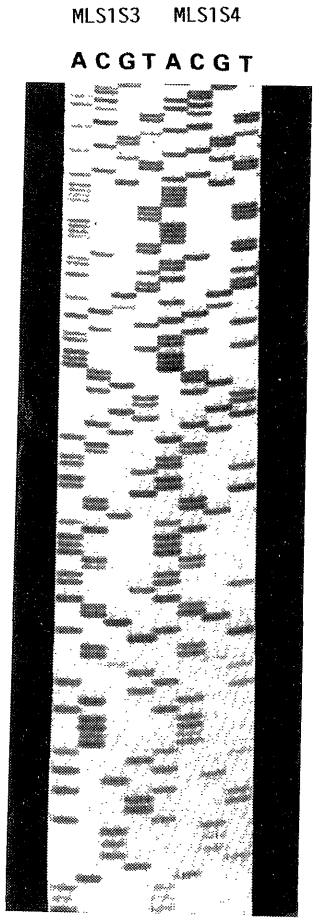

Fig. 4. Radioautograph of a polyacrylamide gel electrophoresis for sequence determination.

Discussion

The oligonucleotide primers used for the PCR in the present study were synthesized according to the nucleotide sequence of the 12S rRNA coding region of the human mtDNA. These primers have been used universally for amplification of the region in mtDNA from a wide variety of species.[4] We have demonstrated that the primers could be used for amplification and analysis of the region of the mtDNA in *L. semifasciata* and that the mtDNA of other species of the sea snakes could be studied in the same manner.

When the nucleotide sequences of six recombinant clones containing the amplified DNA of a single PCR experiment were compared, the sequence differences at 4 positions over 2316 bases were found. This may be due to the limited fidelity of the Tth polymerase in PCR. These synthesis errors could be reduced by employing an appropriate ratio of the enzyme to the template DNA. Nevertheless, the consensus sequence could be deduced from the sequence data of six plasmid clones for each amplified DNA. Furthermore, the nucleotide sequence matched completely among three sepatate sea snake DNA samples. The sequence homology in this region of mtDNA between *L. semifasciata* and human was 69.1%

Direct sequencing of the amplified DNA using internal sequencing primers has been performed and the nucleotide sequence determined by this approach is in agreement with the sequence presented in this paper.

The PCR method has been exploited to other regions of the sea snake mtDNA and the nucleotide sequence analysis of the several regions in the mtDNA of other sea snake species are now in progress. The sequence data should contribute to the understanding of the molecular phylogeny of the sea snake mtDNA.

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