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Cloning and Nucleotide Sequence of Four tRNA Genes in Mitochondrial Genome of Thai Walking Catfish, *Clarias macrocephalus* Günther

Pradit Sangthong and Amnuay Jondeung

ABSTRACT

The *Hind* III digested fragment of Thai walking catfish mitochondrial DNA was cloned and its nucleotide sequence was determined. This fragment, consisting of 668 bp, was composed of partial WANCY region and COI gene. The partial WANCY region was consisted of partial $tRNA^{Trp}$ gene, complete $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$ genes, and origin of light strand replication (O_L). These genes and non-coding sequence (O_L) were the same in their organization as those found in other vertebrates. The partial nucleotide sequence of COI stared with GTG and its inferred amino acids were highly conserved as previously described in other fishes. Interestingly, the intergenic spacer between $tRNA^{Cys}$ and $tRNA^{Tyr}$ was ten nucleotides in length, which might be unique among Clariid species.

Key words: nucleotide sequence, tRNA genes, Thai walking catfish, *Clarias macrocephalus*

INTRODUCTION

Thai walking catfish, *Clarias macrocephalus* Günther, is one of five Clariid species found in Thailand and lives in freshwater swamps, marshes and canals throughout the country and Southeast Asia (Smith, 1945). Thai fish farmers have cultured Thai walking catfish for so long by collecting catfish fry from natural waters such as paddy fields, swamps and canals (Sidthimunka, 1971). After Tongsanga *et al*. (1962) had successfully induced spawning in *C. macrocephalus*, the fingerling production was done by artificial insemination. Since then, the Thai walking catfish has become one of the most popular farming species in Thailand. In 1987, the African catfish, *Clarias gariepinus*,was introduced to Thailand and successfully hybridized with *C. macrocephalus* (Lawonyawut *et al*.,1992). The successful interspecific hybridiazation has resulted in changing the farming species into the hybrid ones. This change also has impact on Thai walking catfish, which is now becoming shortened and threatened. Therefore, it is very inevitable to set up the sustainable conservation and management programs. In making decision regarding the conservation on the genetic resources of this species, an understanding of the amount and distribution of its genetic variation is necessary (Allendorf and Ryman, 1987). The molecular genetic information, such as protein polymorphism, mitochondrial or nuclear DNA variation will be facilitated in order to understand the genetic variability of this species.

The vertebrate mitochondrial genome is a small duplex, covalently closed circular DNA and varies in size ranging from 15-20 kb. All animal mitochondrial genomes contain 37 genes, 2 for

Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

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ribosomal RNAs, 13 for proteins and 22 for tRNAs and one major non-coding region (Boore, 1999). Animal mitochondrial DNA (mtDNA) is haploid and maternally inherited without recombination. MtDNA changes at a rate as much as 5-10 times higher than that of nuclear DNA. This means that genes in mtDNA have higher variability than single-copy genes in nucleus. Therefore, mitochondrial DNA analyses have been widely used to study the population structure and gene flow, hybridization and phylogenetic studies (Moritz *et al.*, 1987) Although the complete mitochondrial DNA sequences of many fish species have been reported but none of *Clarias* species (Boore, 1999).The objective of this study was to sequence a cloned fragment of Thai walking mtDNA in order to obtain a sequence for uses in the further studies. In this report, the first partial nucleotide sequence of WANCY region and of COI gene in mitochondrial genome of Thai walking catfish was presented.

MATERIALS AND METHODS

Mitochondrial DNA isolation.

The mtDNA was isolated by a slightly modified method of White and Densmore III(1992). The fresh eggs of single individual of Thai walking catfish (*Clarias macrocephalus* Günther) was homogenized for 1 stroke in 15 ml TEK (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1.5% KCl) buffer. The homogenate was poured into a 50 ml polypropylene tube and underlayered with 15 ml of 15% sucrose in TEK buffer. The homogenate was then centrifuged at 5,500xg for 10 min. The supernatant was transferred to the new polypropylene tube and centrifuged at 8,000xg for 30 min. The pellet was suspended in 5-10 ml cold EST (100 mM EDTA, 150 mM NaCl, 10 mM Tris-HCl pH 8.0) buffer for 2 cycles and centrifuged at 8,000xg for 15 min. The mitochondrial pellet was resuspended in 500 µl EST and transferred to a microfuge tube. The 18% SDS solution was added to the pellet suspension until final concentration was 2%. All previous steps were carried out at 4° C. The suspension was allowed to stand at room temperature for 10 min. The 250 ml 5M NaCl was added into the microcentrifuge tube and centrifuged at 8,000 g for 15 min. The supernatant was successively extracted by phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform isoamyl alcohol (24:1), respectively. The mtDNA was precipitated with two volumes of ice-cold absolute ethanol overnight and centrifuged at 12,000xg for 10 min at 4°C. After washing with 70% ethanol, the mtDNA was vacuum dried, resuspended in 30 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) buffer and stored at –20°C until use.

Cloning and sequencing.

Isolated mtDNA was completely cleaved with *Hind* III and yielded 5 fragments; 8.8, 3.2, 3.0, 1.1 and 0.6 kb. All *Hind* III digested fragments, covering entire genome of mtDNA, were randomly ligated to pUC 18 with T_4 DNA ligase and transformed into *E. coli* JM 109 as described by Sambrook *et al.* (1989). Transformed colonies were screened for recombinant plasmid by using X-gal color system. Recombinant plasmids were characterized by cleaving with *Hind* III.The inserted fragments were compared to each fragment of intact mtDNA molecules digested with the same restriction enzyme. It was found that there were only two clones, pMmt H500 and pMmt H1000, which contained 0.6kb- and 1.1kb-inserted fragments respectively. The inserted fragment in pMmt H1000 clone was too large to be analysed only with a few reactions. Thus the result will be presented in the next report.

The recombinant, pMmt H500, was sent to Bioservice Unit (BSU) of The National Center for Genetic Engineering and Biotechnology for DNA sequencing. Dye terminator labeling method was applied for sequencing on an automatic sequencer (377 DNA sequencer, PE Biosystem). Nucleotide sequence data were analyzed by the basic BLAST

program available on www.ncbi.nlm.nih.gov/. The tRNA genes were aligned with CLUSTAL W program (Thompson *et al*., 1994) available on http://dot.imgen.bcm.tmc.edu:9331/. The obtained mtDNA sequence of Thai walking catfish was deposited at GenBank data libraries under the accession number AF 322219.

RESULTS AND DISCUSSION

The nucleotide sequence of the L-strand of the Thai walking catfish mtDNA obtained from the clone pMmt H500 is shown in Figure 1. The total length of the partial mitochondrial was 668 bp. The overall base composition of this fragment of L-strand was 39.04%A, 25.75%T, 26.65%C and 18.56%G. An alignment of this fragment with mitochondrial sequences of other organisms deposited in GenBank database revealed that it contained a partial cluster of 5 tRNA genes, origin of L-strand replication (O_L), partial sequence of COI and some intergenic spacers.

A partial cluster of 5 tRNA genes was composed of a partial sequence of $tRNA^{Trp}$ gene and 4 complete genes; $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$ and $tRNA^{Tyr}$. The four tRNA genes were identified by their sequence homologies to other vertebrate tRNAs; their specific anticodons and their potential to fold into cloverleaf secondary structure showed mismatch and variable atypical base-pairing in their stem regions (Figure 2). These tRNAs ranged in size from 69-73 nucleotides. All proposed cloverleaf structures contained 7-8 bp in the amino acid arm, 5-6 bp in the TΨC stem, 5 bp in the anticodon stem and 4-5 bp in the DHU stem. These structures showed variabilities from their counterparts found mtDNA of bichir (Noack *et al.*, 1996) and Japanese sardine(Inoue *et al*., 2000) which were found consistently 7 bp in the amino acid stem, 5 bp in the TΨC stem, 5 bp in the

Figure 1 The partial L-strand nucleotide sequence of Thai walking catfish mitochondrial genome. The direction of transcription is denoted by arrows. Beginning and end of each gene are indicated by a vertical bar (|). The non-coding sequences, origin of L-strand replication (O_L) and intergenic spacer, are overlined with thick and dotted lines respectively. The deduced amino acid sequence for partial COI is shown above the nucleotide sequence (one-letter abbreviation is placed above the first nucleotide of each codon). The *Hind* III retriction sites are underlined.

anticodon stem, and 3-4 bp in the DHU stem. These animal mt-tRNAs are usually smaller and lower G+C content and have more nonstandard base pairs in their stem regions than those of their nuclear counterparts (Brown, 1985). The order of gene arrangement of these 4 tRNAs reported here had similar organization of tRNA genes called WANCY region, a coding region of 5 mitochondrial tRNAs (tryptophan, alanine, asparagine, $O_{L,}$ cysteine and tyrosine)(Seutin *et al.*,1994), which was reported in mitochondrial genome of other fishes(Tzeng *et al*., 1992; Zardoya and Meyer, 1996 ; and Inoue *et al*., 2000).

The origin of light strand replication (O_L) in a non-coding DNA sequence of approximately 30 nucleotides is located in the WANCY

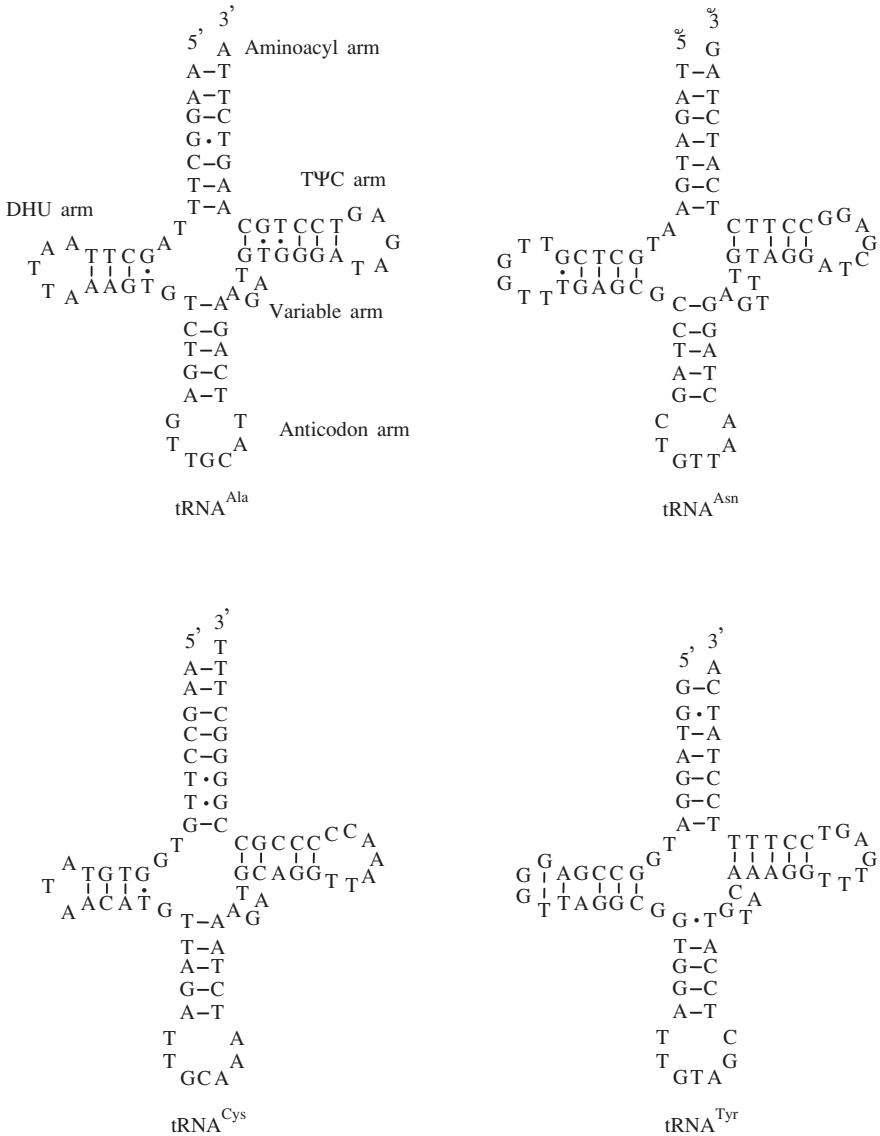

Figure 2 The putative secondary structure of four Thai walking catfish mitochondrial tRNAs; $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$ and $tRNA^{Tyr}$, based on sequence of Figure 1.

region(Seutin *et al.*,1994). In Thai walking catfish mtDNA fragment, the O_L found between the $tRNA^{Asn}$ and $tRNA^{Cys}$ and is 35 nucleotides in length. The O_L sequence had capability to form a stable stem-loop structure with 11 bp in the stem, 13 nucleotides in the loop (Figure 3). The four nucleotides of the O_L stem were parts of $tRNA^{Cys}$ gene. It was slightly different from that of lungfish mtDNA which about half of O_L stem shared with $tRNA^{Cys}$ gene (Zardoya and Meyer, 1996). The Thai walking catfish O_L also had a conserved motif, 5'-GCCGG-3', at base with $tRNA^{Cys}$ gene. This conserved motif of O_L has been also reported in Atlantic cod, loach, bichir and human (Noack *et al.*, 1996; Tzeng *et al.*, 1992 and Anderson *et al.*, 1981). The most interesting character of Thai walking catfish O_L is a A-T rich sequence in the loop, which has been reported in bichir (Noack *et al.*, 1996), African clawed frog (Roe *et al.*,1985) and human (Anderson *et al.*, 1981). The role of the thymine rich sequence in loop region in the human is shown to be involved in synthesis of RNA primer for light-strand replication process (Wong and Clayton, 1985).

The partial sequence of COI, as shown in Figure 1, is 305 nucleotides in length. The derived amino acid sequence of a part of COI gene, coding for cytochrome c oxidase subunit I, is shown in

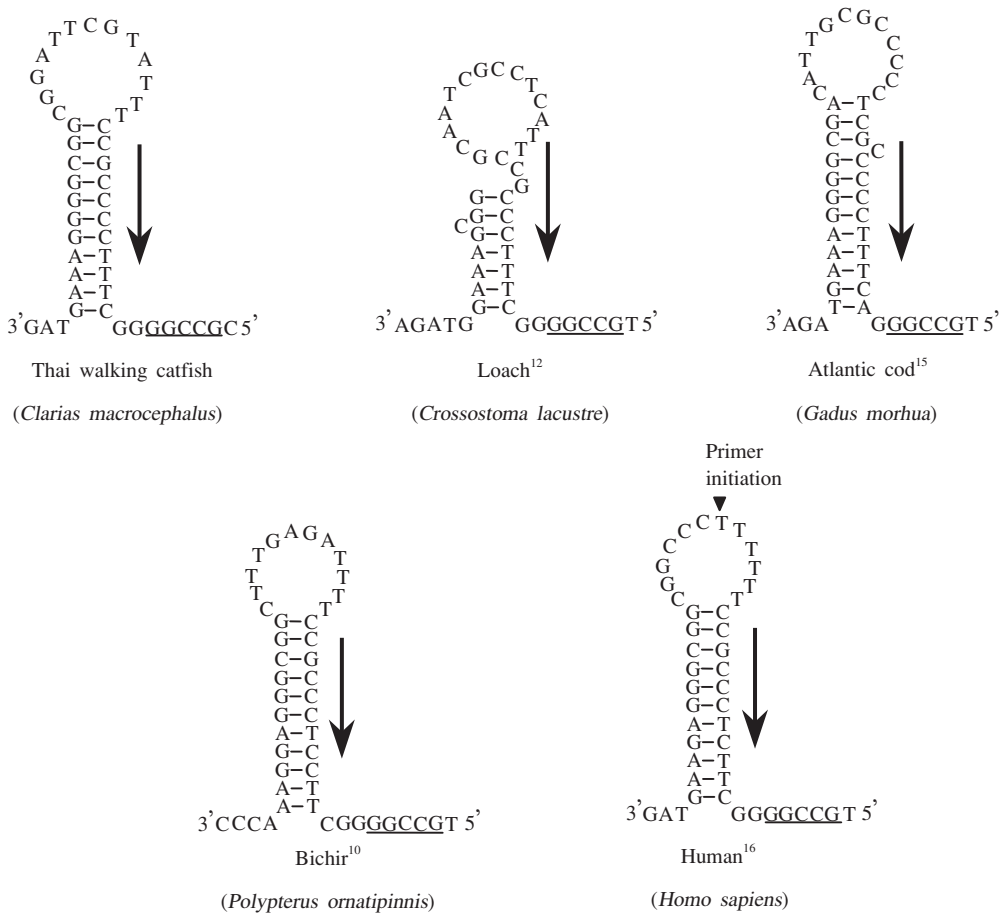

Figure 3 The stem-loop structures for the origin of light strand replication (O_L) of Thai walking catfish mtDNA and other vertebrates. The underlined sequence represents conserved motif.

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            10        20        30        40        50        60
Mac MTITRWFFSTNHKDIGTLYLVFGAWAGMVGTALSLLIRAELAQPGALLGDDQIYNVIVTA  60
Cod .A.....................................S.................  60
Loa .A.....................................N.................  60
Tro .A.....................................S.................  60

            70        80        90       100
Mac HAFVMIFFMVMPIMIGGFGNWLVPLMIGAPDMAFPRMNNMS  101
Cod ............L.........I..................  101
Loa ............L.............H..........  101
Tro ....................I..................  101
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Figure 4 The inferred amino acid sequence of partial COI gene of Thai walikng catfish (Mac) and those of other fishes: Atlantic cod (Cod), Loach (Loa), and Trout (Tro). A dot indicates amino acid similarity.

Figure 4. The COI gene starts with the initiation codon of GTG. Normally, this triplet codon specifies valine within open reading frame. GTG as initiation codon has been previously reported in many fish species such as freshwater loach (Tzeng *et al*., 1992), lungfish (Zardoya and Meyer, 1996), bichir (Noack *et al.*, 1996), deep sea fish (Miya and Nishida, 1999) and Japanese sardine (Inoue *et al.*,2000), but not in Atlantic cod (Johansen *et al.*,1990), which use GUG as the initiation codon for the COI gene. The inferred amino acid sequence of partial COI is also compared to those sequences of cod, loach and trout (Figure 4). It was found that the homologies of TW catfish COI compared to that of other fish ranges from 96 to 97%. This shows that COI polypeptide sequence is highly conserved as previously described in Atlantic cod, loach and trout (Johansen *et al*., 1990; Tzeng *et al.*, 1992; Zardoya *et al*. 1995)

Interestingly, the intergenic spacer between $tRNA^{Cys}$ and $tRNA^{Tyr}$ is 10 nucleotides in length. It has been reported that the intergenic spacers in many fish species are absent or small number of nucleotides (Miya and Nishida, 1999; Inoue *et al.*, 2000) Long spacer has been found between $tRNA^{Thr}$ and $tRNA^{Pro}$ genes of 8 cod fish species which varies in size ranging form 25 to 99 bp and their variation can be determined both at intraspecific and interspecific levels(Bakke *et al*., 1999) Thus, the intergenic spacer found here might be a unique feature of the Clariid species.

CONCLUSION

The mtDNA of Thai walking catfish was isolated from freshly ripen eggs, cleaved with *Hind* III and cloned into pUC18. A recombinant clone, pMmt 500 contaning about 0.6kb-inserted fragment, was screened by using X-gal system and sent for sequencing of its inserted fragment. The inserted mtDNA fragment was found to be 668 bp in length and composed of a partial WANCY region and a partial COI gene. The sequence was analyzed and discussed comparatively with mitochondrial sequences of other organisms.

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