

Cytochrome C Oxidase Subunit-1(COX1) Gene in Tilapia (*Oreochromis Niloticus*): its Cloning and Characterization

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Abstract The current study aimed to identify cytochrome c oxidase subunit 1 (ONCOX1) in Tilapia (*Oreochromis niloticus*) immunized by formalin-killed *Flavobacterium columnarae*. Suppressive subtractive hybridization (SSH) was utilized to construct a cDNA library and a semi-quantitative RT-PCR analysis used to examine *Oreochromis niloticus* cytochrome c oxidase subunit 1 (COX1) gene expression. The complete sequence of the COX1 has been deposited in the GenBank Database and assigned the accession number (GE647880). COX1 cDNA is composed of 1139 bps with a 1107 bps open reading frame, the predicted gene product is 369 amino acid with molecular weight of 40.16 kDa. The amino acid sequence revealed high identity with subunit 1 from *Oreochromis mossambicus*. Compared to β -actin, the semi-quantitative RT-PCR revealed that ONCOX1 expressed in tissues of stimulated fish as up-regulated gene suggesting that this member of COX genes is probably involved in the general immune response against the pathogenic bacteria.

Keywords Tilapia, Cytochrome C Oxidase , Gene Expression, Protein Characterization

1. Introduction

Cytochrome c is a small, highly conserved, heme- containing bifunctional protein; it has been studied extensively, not only for its role in electron transport, but also for its role in apoptosis, it releases from the mitochondria in response to specific apoptotic stimuli (Wang X., 2001). Cytochrome c oxidase (COX) is an oligomeric enzymatic complex located in the inner membrane of mitochondria which consists of three large primarily catalytic subunits encoded by the mitochondrial genome and 10 smaller subunits encoded by nuclear genes. The nuclear encoded subunits are thought to modulate enzyme function. In vertebrates, COX is composed of 13-subunits, and a further 30 proteins are required for proper COX assembly (Ettickan *et al*, 2004). In eukaryotes this enzyme complex is located in the mitochondrial inner membrane. It is considered to be a major site of regulation of mitochondrial oxidative phosphorylation. This rate-limiting enzyme is also implicated in the production of reactive oxygen species (ROS) under oxidative stress conditions (Lee *et al*, 2001, 2003 and Vijayasarthy *et al.*, 2003). *COX1* encodes an important enzyme involved in the oxidation phosphorylation pathway and thus energy production. In an attempt to characterize differential gene expression during cDNA library construction from tilapia immunized by *F. columnarae*, we could recognize ONCOX1 as up-regulated

gene. The specific hypothesis for this study is that gene characterization and detectable changes in gene expression directed by certain pathobiological conditions may identify genetic information that are important for understanding the host-pathogen relationship and host immune responses., thus in this paper we summarize the identification of a cytochrome c oxidase subunit 1 gene utilizing available protein structure prediction methods based on the predicted protein sequence. The sequence we have identified and its predicted corresponding structure is the first functional annotation for this family of proteins in *O.niloticus*.

2. Material and Methods

Tissue preparation and RNA extraction

Oreochromis niloticus 12 individuals were obtained from Wuhan, China. The fish were held in the laboratory, acclimatized in recirculating freshwater tank at room temperature under natural photoperiod for a week and fed with commercial pellets at a daily ration of 3% of their body weight. They were divided into two groups, the first were stimulated for four days each with intraperitoneal injection of 0.5 ml 10^7 formalin killed *Flavobacterium columnarae*, the other group were injected with PBS and used as a control. Two *F.colmnarae* injected tilapia, 24.5 cm and 27 cm in fork length were dissected, gills, liver, spleen, head kidney and intestine were dissected out for the construction of the infected group subtracted cDNA library. One control (PBS injected fish) 23.7 cm in fork length was used also for the subtracted library with the mentioned organs dissected out. Total RNA was prepared for RT-PCR using Trizol Reagent

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(Invitrogen, USA). The mRNA was then isolated from total RNA using the poly Atract Isolation System (Promega). Concentration of mRNA was determined using a spectrophotometer.

SMART cDNA synthesis and Suppressive subtraction hybridization (SSH)

Complimentary DNA was synthesized and amplified using a CLONTECH cDNA Synthesis Kit by following the the manufacture's instruction. The mRNA obtained from tester and driver samples was subjected to SSH and selective PCR amplification using PCR-select cDNA subtraction kit to construct a cDNA library.

Cloning and screening for differential expression by dot blotting and sequence analysis

secondary PCR products of stimulated and unstimulated directly inserted into pGEM-T using a pGEM-T Easy Vector System (Promega), which was then transformed into *Escherichia coli* and screened by the white colonies which were selected and amplified with nested PCR primer 1(5'-TCGAGCGGCCGCCCCGGGCAGGT-3') and nested PCR primer 2(5'-AGGGTGGTCGCGGCCGAGGT-3'). 3 µl of PCR product were denatured in 3 ml of 0.5 M NaOH. Two identical H-bond N⁺ nylon membranes were prepared by loading 1 µl of denatured PCR product of each clone on the same location, after 5 min. neutralization in 0.5 M Tris-HCl (pH7.5), the membranes were baked for 30 min at 120 °C to cross link the cDNAs.

Forward-subtracted cDNAs were digested with RsaI and labeled as probes with digoxigenin using a DIG High Prime system (Boehringer Mannheim) by following the manufacture's instruction. Positive clones were sequenced using dedoxy chain sequencer (ABI Applied Biosystems Model 337).

Sequence and phylogenetic analysis

Sequences were compared with the sequences in the database using the BLASTX program at the web server of the National biotechnology information. Protein prediction was performed using software at the ExPASy Molecular Biology Server (<http://expasy.pku.edu.cn>) and SAPS program (Statistical Analysis of Protein Sequences). Sequences were aligned, employing the distance matrix; a distance-joining tree was constructed and a Phylogenetic analysis was carried out for the deduced amino acid sequences of cytochrome c oxidase subunit 1(COX1) using Clustal W version 1.83(Thompson *et al.* 1994), Sequence database alignments and comparisons were done with the BLAST family of programs (blastx,) against database specifications of non-redundant protein, SWISS-PROT which were available from the BLAST website at the National Center for Biotechnology Information webserver, (<http://www.ncbi.nlm.nih.gov/blast/>) (Altschul *et al.* 1997). A signal peptide was predicted using Signal P 3.0 and analysis for transmembrane helices was applied using SOSUI dumbbell server.

Semi - quantitative RT - PCR for tissue expression of COX1

Total RNA from different mixed tissues was treated with DNase, 2 µg RNA was reverse transcribed with M-MLV reverse transcriptase using hexanucleotides (Promega) to prim the reaction. The first strand cDNA was used as templates for RT-PCR with a pair of COX1 specific primers designed, forward 5'- TTGGACACCTGAAGT-3' and reverse: 5' GTGGCGGAAGTAAAGT3'.

The PCR cycling parameters were one cycle of 94°C for 5 min, 35 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 1 min, with a final extension step of 72°C for 10 min. The RT-PCR products were analyzed by electrophoresis on 2% agarose gel with PCR products derived from beta actin of the infected and non infected tilapia as controls, documented with documentation system. Semi-quantitative assessment of mRNA levels were determined by quantifying the band's intensity of PCR products using GeneTools in the gene expression relative to beta actin were tested between infected and uninfected fish using t-test.

3. Results

Isolation of differentially expressed clones

The comparison of pooled RNA samples from *Oreochromis niloticus* immunized by *Flavobacterium columnarae* against unimmunized healthy *Oreochromis niloticus* yielded multiple differentially expressed clones; some were suggested as up-regulated genes in the process of immunization as revealed by dot blot analysis and RT-PCR.

Characterization of cDNA sequence of ONCOX1

The complete sequence of the COX1 has been deposited in the GenBank Database (<http://www.ncbi.nlm.nih.gov/Genbank/>) and was assigned the GenBank nucleotide accession number (GE647880). Blast queries of the sequenced nucleotides of COX1 demonstrated that the deduced amino acid sequence contains 1139 nucleotide bps with 1107 bps open reading frame coding for a protein of 369 aa and molecular weight 40.16 kda (Fig. 1).

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1  TTASAKLAQL QVDDRGRGRG THAGPSVDLT IFSHLGVS SILGAINFIT TIINMKPPAI
61  SQQTPLFW SVLITAVLLL LSLFVLAAGI TILLTDRLN TTFDFAGGG DPILYQLFF
121 FGHPEVYILI LFGFGIISHI VAYYAGKKEP FGVMGMWAM MAIGLLGFIV AHMFTVGMD
181 VDRAYFTSA TIIIIAIPGV KVFSLATLHG GAIKETELLA LGFIFLFTVG GLTGIVLANS
241 SLDIMLHDTY YVVAHFHYVL SIGAVFAIVA GFVHPLFSG YTLHDTTKIH FGVMFIGVNL
301 TFFPHISWDW QECFVGTCPA AARNLRIPGY RARSYHGSKL FSGNGYPLTN SHQTLQTRKH
361 KGTWGPYIA

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Figure 1. Deduced amino acid sequences of ONCOX1

Protein structure prediction

Through search of available sequence databases showed that the putative ONCOX1 sequence is homologous to other known cytochrome c oxidases with varying levels of sequence identity and high similarity with *Oreochromis mossambicus* (accession no.YP_272017) and also to human (accession no.BAA07292) and several vertebrates COX1. A signal peptide was predicted using Signal P 3.0, deduced amino acids analysis confirmed that this sequence has no signal peptide, the signal peptide probability=0.026, signal

anchor probability: 0.126 and the max cleavage site probability: 0.017 between bps 44 and 45. Average of hydrophobicity = 0.582114. The revealed isoelectric point (pI) of ONCOX1 = 9.51.

Multiple methods of transmembrane prediction were used to enable a consensus confirmation of predicted transmembrane helices. The TM region search for ONCOX1 by SOSUI analysis program beta version suggests that ONCOX1 is a membrane protein with 8 transmembrane helices(TM). Transmembrane helices were found in the positions 29-53, 72-95, 126-145, 152-171, 192-211, 218-237, 258-282 and 289-306 as showed in table 1.

Alignment analysis and phylogenetic tree construction

A Basic Genebee ClustalW 1.83 was performed on a variety of Cox1 genes from *Engraulis japonicas*; *Pylodictis olivaris*; *Homo sapiens*; *Mus musculus*; *Oreochromis mossambicus* and *Oreochromis niloticus* (accession nos. NP_203729; AAL91550; BAA07292; CAD54434; YP_272017 and GE647880 respectively). Fig.1. *Oreochromis niloticus* shares an overall identity of 89.2 - 99% with other known vertebrates. Using the Clustal W and the GeneBee programs, a phylogenetic tree was generated (Fig.2, 3).

Expression of HSP9 in *Oreochromis niloticus*

ONCOX1 expression studied by RT-PCR in mixed tissues from infected and uninfected control fish was detected only in the infected tilapia (Fig.4).

4. Discussion

Suppression subtractive hybridization (SSH) has led to the enrichment of specific expression genes as the non-specific expression of beta actin was reduced to about 2^{15} time lower after subtraction, suggesting that non-specific expressed genes have been avoided and immunization- specific genes have been enriched efficiently in this study. The identification of new genes involved in the bacterial immunization provides the foundation for further research on the immunological interaction between host and the bacteria. In this study, cDNA library construction in case of *O. niloticus* immunized by *F. columnarae* introduced some new immune genes, one of them was ONCOX1.

dynamic change in an environment, disease hazards and the host-pathogen relationship provided signals for identifying differentially expressed genes which may play an im-

portant role in the immune system responses. During construction of cDNA library from *O. niloticus* immunized by *F. columnarae*, we could identify and characterize cytochrome c oxidase subunit 1 gene as up-regulated gene.

ONCOX1 sequence was found to be homologous to other known cytochrome c oxidases with varying levels of sequence identity and high similarity with *Oreochromis mossambicus* (accession no.YP_272017) and also to human (accession no.BAA07292) and several vertebrates COX1, it appears to have structural features similar to the largest subunit of the heme/copper-requiring cytochrome c.

The prediction of O-GalNAc (mucin-type) for glycosylation sites in ONCOX1 proteins indicated the absence of any glycosylation sites. Multiple methods of transmembrane prediction were used to enable a consensus confirmation of predicted transmembrane helices. The TM region search for ONCOX1 by SOSUI analysis program beta version suggests that ONCOX1 is a membrane protein with 8 transmembrane helices(TM). Transmembrane helices were found in the positions 29-53, 72-95, 126-145, 152-171, 192-211, 218-237, 258-282 and 289-306 as showed in table 1. The objective of this TM search step was to identify and map out regions of transmembrane helices to confirm our sequence database based hypothesis of the putative sequence being identified as a cytochrome c oxidase subunit I. Cytochrome c oxidases are known to have these transmembrane regions and the identification of these regions served as a confirmatory step in gauging the validity of the predicted structure (Mohamed *et al.*, 2003). The observation from the transmembrane prediction step is consistent with the subunit I of most other haem copper oxidases and acts as confirmatory data for correctness of the protein fold generated by the analysis program.

A final taxonomic system for the animal kingdom will probably include at least 10 million species partitioned among more than a million genera (Paul *et al.*, 2003). Given such high diversity, there is a growing realization that it is critical to seek technological assistance for its initial description and its subsequent recognition (Godfray 2002; Blaxter 2003). Recent investigations have suggested the feasibility of creating identification systems reliant on the analysis of sequence diversity in small segments of DNA (Tautz *et al.* 2003). Hebert *et al.* (2003) focused this discussion by proposing that a DNA barcoding system for animal life could be based upon sequence diversity in cytochrome c

Table 1. Analysis of COX1 transmembrane amino acid sequence (SOSUI dumbbell server)

No.	N terminal	transmembrane region	C terminal	type	length
1	31	IFSLHLAGVSSILGAINFITTH	53	Secondary	23
2	73	LITAVLLLLSLPVLAAAGITILLT	95	Primary	23
3	126	VYILILPGFGIISHIVAYYAG	146	Primary	21
4	153	YMGMVWAMMAIGLLGFIVAHHMF	175	Primary	23
5	188	TSATIIIAIPTGVKVFSLATLHG	210	Secondary	23
6	219	LALGFIFLFTVGGTLTGIVLANSS	241	Primary	23
7	257	HYVLSIGAVFAIVAGFVHFPLFS	279	Primary	23
8	286	TTKIHFVGMFIGVNLTFPHISW	308	Secondary	23

Engraulis	MAITRWFFSTNHKDIGTLYLFGAWAGMVGTSLLIRAEISQPGALLGDDQYNNVITA
Pylodictis	MTITRWFFSTNHKDIGTLYLFGAWAGMVGTSLLIRAEISQPGALLGDDQYNNVITA
Homo	MFADRWFFSTNHKDIGTLYLFGAWAGMVGTSLLIRAEISQPGALLGDDQYNNVITA
Mus	MFTNRWFFSTNHKDIGTLYLFGAWAGMVGTSLLIRAEISQPGALLGDDQYNNVITA
Oreochromis	MAITRWFFSTNHKDIGTLYLFGAWAGMVGTSLLIRAEISQPGALLGDDQYNNVITA
niletilapia	-----
Engraulis	HAFVMFFMVMPIGGFGNWLPLMIGAPDMAFPRMNNMSFWLLPSSFLLLASSGVEA
Pylodictis	HAFVMFFMVMPIGGFGNWLPLMIGAPDMAFPRMNNMSFWLLPSSFLLLASSGVEA
Homo	HAFVMFFMVMPIGGFGNWLPLMIGAPDMAFPRMNNMSFWLLPSSFLLLASSGVEA
Mus	HAFVMFFMVMPIGGFGNWLPLMIGAPDMAFPRMNNMSFWLLPSSFLLLASSGVEA
Oreochromis	HAFVMFFMVMPIGGFGNWLPLMIGAPDMAFPRMNNMSFWLLPSSFLLLASSGVEA
niletilapia	-----TTASAKLACIQVD-----DR
Engraulis	GAGTGWTVYPLAGNLAHAGASVDLTFSLHLAGVSSILGAINFITTHNMKPPAISQYQ
Pylodictis	GAGTGWTVYPLAGNLAHAGASVDLTFSLHLAGVSSILGAINFITTHNMKPPAISQYQ
Homo	GAGTGWTVYPLAGNLAHAGASVDLTFSLHLAGVSSILGAINFITTHNMKPPAISQYQ
Mus	GAGTGWTVYPLAGNLAHAGASVDLTFSLHLAGVSSILGAINFITTHNMKPPAISQYQ
Oreochromis	GAGTGWTVYPLAGNLAHAGASVDLTFSLHLAGVSSILGAINFITTHNMKPPAISQYQ
niletilapia	GRGRG-----THAGPSVDLTFSLHLAGVSSILGAINFITTHNMKPPAISQYQ
Engraulis	TPLFVWSVLITAVLLLSLPVLAAGITMLTDRNLNTFFDPAGGGDPILYQHLFWFFGH
Pylodictis	TPLFVWSVLITAVLLLSLPVLAAGITMLTDRNLNTFFDPAGGGDPILYQHLFWFFGH
Homo	TPLFVWSVLITAVLLLSLPVLAAGITMLTDRNLNTFFDPAGGGDPILYQHLFWFFGH
Mus	TPLFVWSVLITAVLLLSLPVLAAGITMLTDRNLNTFFDPAGGGDPILYQHLFWFFGH
Oreochromis	TPLFVWSVLITAVLLLSLPVLAAGITMLTDRNLNTFFDPAGGGDPILYQHLFWFFGH
niletilapia	TPLFVWSVLITAVLLLSLPVLAAGITMLTDRNLNTFFDPAGGGDPILYQHLFWFFGH
Engraulis	PEVYILILPGFGMISHVAYYAGKKEPFYMGVMWAMMAIGLGFVWAHMFVGMVDV
Pylodictis	QSMVILILPGFGMISHVAYYAGKKEPFYMGVMWAMMAIGLGFVWAHMFVGMVDV
Homo	PEVYILILPGFGMISHVAYYAGKKEPFYMGVMWAMMAIGLGFVWAHMFVGMVDV
Mus	PEVYILILPGFGMISHVAYYAGKKEPFYMGVMWAMMAIGLGFVWAHMFVGMVDV
Oreochromis	PEVYILILPGFGMISHVAYYAGKKEPFYMGVMWAMMAIGLGFVWAHMFVGMVDV
niletilapia	PEVYILILPGFGMISHVAYYAGKKEPFYMGVMWAMMAIGLGFVWAHMFVGMVDV
Engraulis	TRAYFTSATMIAIPTGVKVSFLATLHGGAIKWEPTLWALGFILFTVGGLTGIVLAN
Pylodictis	TRAYFTSATMIAIPTGVKVSFLATLHGGAIKWEPTLWALGFILFTVGGLTGIVLAN
Homo	TRAYFTSATMIAIPTGVKVSFLATLHGGAIKWEPTLWALGFILFTVGGLTGIVLAN
Mus	TRAYFTSATMIAIPTGVKVSFLATLHGGAIKWEPTLWALGFILFTVGGLTGIVLAN
Oreochromis	TRAYFTSATMIAIPTGVKVSFLATLHGGAIKWEPTLWALGFILFTVGGLTGIVLAN
niletilapia	TRAYFTSATMIAIPTGVKVSFLATLHGGAIKWEPTLWALGFILFTVGGLTGIVLAN
Engraulis	SSLDIVLHDTYVVAHFHYVLSMGAVFAIVAGFVHWPFLFTGYTLHSWTKIHFGVMFVG
Pylodictis	SSLDIVLHDTYVVAHFHYVLSMGAVFAIVAGFVHWPFLFTGYTLHSWTKIHFGVMFVG
Homo	SSLDIVLHDTYVVAHFHYVLSMGAVFAIVAGFVHWPFLFTGYTLHSWTKIHFGVMFVG
Mus	SSLDIVLHDTYVVAHFHYVLSMGAVFAIVAGFVHWPFLFTGYTLHSWTKIHFGVMFVG
Oreochromis	SSLDIVLHDTYVVAHFHYVLSMGAVFAIVAGFVHWPFLFTGYTLHSWTKIHFGVMFVG
niletilapia	SSLDIVLHDTYVVAHFHYVLSMGAVFAIVAGFVHWPFLFTGYTLHSWTKIHFGVMFVG
Engraulis	VNLTFPPQHFLGLAGMPPRYSDYDAYT-LWNTVSSIGSLISLAVIMFLIWEAFAA
Pylodictis	VNLTFPPQHFLGLAGMPPRYSDYDAYT-LWNTVSSIGSLISLAVIMFLIWEAFAA
Homo	VNLTFPPQHFLGLAGMPPRYSDYDAYT-LWNTVSSIGSLISLAVIMFLIWEAFAA
Mus	VNLTFPPQHFLGLAGMPPRYSDYDAYT-LWNTVSSIGSLISLAVIMFLIWEAFAA
Oreochromis	VNLTFPPQHFLGLAGMPPRYSDYDAYT-LWNTVSSIGSLISLAVIMFLIWEAFAA
niletilapia	VNLTFPPQHFLGLAGMPPRYSDYDAYT-LWNTVSSIGSLISLAVIMFLIWEAFAA
Engraulis	KREVASVELTITNVEWLHGCPPPYHTFEPAFVQVK-----
Pylodictis	KREVASVELTITNVEWLHGCPPPYHTFEPAFVQVK-----
Homo	KREVASVELTITNVEWLHGCPPPYHTFEPAFVQVK-----
Mus	KREVASVELTITNVEWLHGCPPPYHTFEPAFVQVK-----
Oreochromis	KREVASVELTITNVEWLHGCPPPYHTFEPAFVQVK-----
niletilapia	KREVASVELTITNVEWLHGCPPPYHTFEPAFVQVK-----

Figure 2. The alignment of COX1 deduced amino acid sequences of from *Engraulis japonicas*; *Pylodictis olivaris*; *Homo sapiens*; *Mus musculus*; *Oreochromis mossambicus* and *Oreochromis niloticus* (accession nos. NP_203729; AAL91550; BAA07292; CAD54434; YP_272017 and GE647880 respectively). Identical amino acids are indicated with an asterisk, similar amino acids are shaded. Dashes are gaps generated by alignment

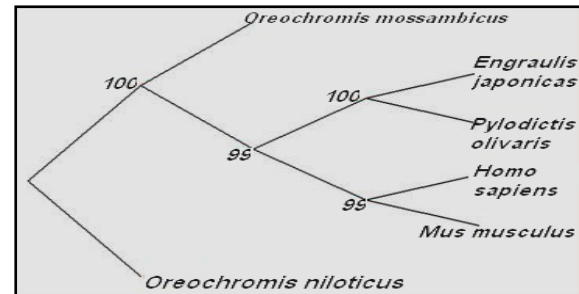


Figure 3. phylogenetic distance tree showing the relationship among the COX1 from different vertebrates



Figure 4. Tissue specific expression revealed by RT-PCR in *Oreochromis niloticus*; 1=βactin 2=immunized fish, 3= unimmunized fish

Paul *et al.*, 2003 in their study on COX1 divergences among closely related species indicated that an identification system for animal life based on the COI gene will be highly effective, although COX1 divergences appear too low to regularly enable species diagnosis within the cnidarians, generic-level identifications in these organisms remain a prospect, as their study has established that species-level identifications are ordinarily achieved, COI analysis actually provides a taxonomic system that is chasing the last digit of animal diversity.

The mRNA expression of mixed tissues of *F. columnare* - immunized *O. niloticus* indicated that the COX1 enzyme coding gene was highly expressed in the immunized in contrast to unimmunized fish, thus it is suggested to be included in the immune response against *F. columnare*.

Cytochrome *C*, which is a component of the mitochondrial electron transfer chain releases from the mitochondria in response to specific apoptotic stimuli (Wang X., 2001). It initiates caspase activation when released from mitochondria during apoptosis (Liu *et al.* 1996), Skulachev V.P.(1998) in his study on apoptosis has revealed that cytochrome *c* is a pro-apoptotic factor. It is released from its places on the outer surface of the inner mitochondrial membrane at early steps of apoptosis and, combining with some cytosolic proteins, activates conversion of the latent apoptosis-promoting protease pro-caspase-9 to its active form. Thus from this investigation, it is suggested that COX1 may be included in the immune response against bacterial infection, the higher level of expression may also imply its role as an apoptotic inducer.

5. Conclusions

It could be concluded that killed *F. columnare* did stimulate the immune response in the host fish, tilapia, some immune genes could be identified and characterized, one of these genes is COX1, which may have a role and included in

the immune response to bacterial infection, thus the present study provides some information for further research on the structure of COX1 gene as an immune gene in relation to bacterial infection.

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