Mitochondrial Cytochrome C Oxidase Subunit 1 (cox 1) Gene Sequence of the Hymenolepis Species.


Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt, kandil_om@yahoo.com*

Abstract: In the current study, Mitochondrial Cytochrome c oxidase gene especially codons within subunit 1 (cox1) of H. diminuta and H. nana Egyptian isolates from two stages (adult worms and eggs) and hosts origin (human and rat) were amplified, sequenced and aligned. PCR products were approximately 700 bp, 702 bp and 715 bp of H. nana rat isolates, H. diminuta rat isolates, and H. nana human isolates, respectively. Moreover, despite their host susceptibility differences they all gathered in one cluster with three genbank published isolates of H. nana; AB033412.1, AB494472.1 and AY121842.1, forming one clade with 100% similarity, which was non significantly decreased on internal nodes. In addition, clearly far away from H. diminuta published sequence AB033412.1 who's assumed to be genetically closely related to Egyptian H. diminuta than all other H. nana isolates. Both Egyptian murine isolates of Hymenolipidid; H. diminuta and H. nana, were closer to each other than being to H. nana of human origin. The annotated sequences of Egyptian isolates were deposited in GenBank under the following accession numbers; H. diminuta (GU433102), H. nana rat isolate (GU433103), and H. nana human isolate (GU433104). Finally, the development of effective control strategies will only be possible if complete understanding of the epidemiology of infection is elucidated.

Keywords: Hymenolepidid, Phylogeny, Cytochrome c oxidase subunit 1 gene (cox1), Sequencing.

1. Introduction: Hymenolipis nana and H. diminuta are the most common cestodes in humans, mice, domestic and wild rats (Macko and Hanzelova 2008). It is believed that infections with Hymenolipis spp., in general, may have been under diagnosed due to sporadic egg shedding (Thompson et al. 2001; Raether and Hanel 2003). Since isolates of H. nana infecting humans and rodents are morphologically identical, the only way they can be reliably distinguished is comparing the parasite in each host using molecular techniques (Macnish et al. 2002a, b).

Mitochondrial (mt) genomes are small (usually less than 20000 bp), circular, and maternally inherited (Boore 1999). In addition to high copy-number per cell which has made them attractive and more tractable targets for characterization, population genetic and phylogenetic studies (Hu et al. 2004; McManus et al. 2004). Regions within the mitochondrial DNA (mtDNA) have been proven useful in biology, epidemiology and diagnosis of several parasitic infections of human and veterinary importance (Ngarmamonpitrat et al. 2005; Ando et al. 2006). Methods used to obtain data from flatworm mt genomes have included DNA sequencing, restriction fragment length polymorphism (RFLP) analysis and single-strand conformation polymorphism (PCR-SSCP) (Boore and Brown 1998; Avise, 2000).

Intraspecific sequence variation in coding portions (genes) of the mt genomes seems to range from small to moderate, especially when compared with interspecific variation that have demonstrated the deep separations among strains of same species (Littlewood et al. 2008).

Complete or near-complete mtDNA sequences are available for 12 species of parasitic flatworms; six cestodes including Taenia crassiceps (Le et al. 2000), Echinococcus multilocularis (Nakao et al. 2000) and Hymenolepis diminuta (von Nickisch-Rosenegk et al. 2001). Cytochrome c oxidase (COX) is a 13-subunit protein complex located on the inner mitochondrial membrane that catalyzes electron transfer, proton translocation processes, production of up to 95% of the energy of eukaryotic living cells (Saraste 1999; Johnston 2006), thus directly influence metabolic performance. mt cox sub unit 1 is the most highly conserved among 3 genes coding for cytochrome oxidase, therefore has been employed in several phylogenetic studies (Traversa et al. 2007).

DNA sequencing of informative regions within the gene encoding for the COX1 protein have emphasized specific comparative aspects without yet making a detailed genome description but revealed data for basic and applied potential differential studies on Hymenolepis spp. determining host
specificity and transmission patterns (Macnish et al. 2003). Therefore, allow more appropriate approach for control of endemic infestations in Egypt, particular where rodent's population is above control limits and hygienic measures are not strictly applied. Furthermore, for diagnostic purposes since using techniques able to overcome inherent limits of the classical approaches (Constantine 2003; Thompson et al. 2004). Epidemiologically, despite this infection is a hand-to-mouth rote that in general not very pathogenic, however it is extremely difficult to be controlled (Littlewood et al. 2008). Till now education in hygiene is probably the only practical way to reduce the incidence in addition to rodent's eradication (Behera et al. 2008). The genotyping of Hymenolepis isolates in different hosts will help in determine host specificity and transmission patterns and thus allow more appropriate approach to control infections in endemic communities. From a public health perspective, a better understanding of the transmission dynamics of a parasite species previously believed to be infective only to rodents will be required to answer questions about the potential for transfer of this parasite to humans and/or animals.

Since control of parasitic disease is dependent on the rapid and accurate detection of causative agents this necessitated traditional techniques being complemented by molecular tools that provide predictive data on genetic variation in and among parasites (Thompson et al. 2004). Thus the present work aims is to characterize, for the first time, partial sequences of cox1 genes of H. diminuta and H. nana Egyptian isolates to promote basic knowledge on their mtDNA composition, to assess the sequence variation level within local Hymenolepidid from different sources, different developmental stages (adult worms and eggs) and hosts origin (human and rat), and to discuss the potential benefits of such molecular information as record sheets for ecological, epidemiological, transmission and host-parasite interaction and as diagnostic approach of infection in Egypt.

2. Materials and methods
Parasites Samples:
H nana eggs were obtained from infected humans in Endemic Diseases institute. Approximately 2000 H. nana eggs were inoculated into 5-week-old male white mice (Movsesyan et al. 2008). Adult worms were dissected from the small intestine immediately 14 days post-inoculation (Tanowitz et al., 2001). H. diminuta worms were obtained from naturally infected norvegicus rat from Abu Rawash, Giza, Egypt. Rats were killed by cervical dislocation and entire small intestine was removed from gut. The worms and eggs washed repeatedly in phosphate buffered saline (PBS) and stored at -80 °C until used for DNA extraction.

Isolation of DNA from Adult Worms and Eggs
Templates DNA were purified from H. nana and H. diminuta using QI Amp tissue purification kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions (Macnish et al. 2002a). DNA was eluted in 200 µl Tris-EDTA (TE) buffer and 1 µl of the extract was added to the polymerase chain reaction mix. Single adult worm and/or eggs for each isolate were used for DNA extraction.

Oligonucleotide Primers Design
Entire mt genomes of the following species were aligned Hymenolepis diminuta (accession number AB033412.1), Taenia crassiceps (accession number NC_002547), T. solium (accession number NC_004022), T. asiatica (accession number NC_004826), Echinococcus granulosus (accession number NC_008075), and E. multilocularis (accession number NC_000928); and annotated sequence of Hymenolepis nana (accession number AF314223.1) (Nakoo et al. 2000, 2002; von Nickisch-Rosenegk et al. 2001). It was not deemed necessary to include all the available sequences from Taenia or Echinococcus as conservation of alignable positions between genera and being > 30% GC was more important for PCR primer design. PCR primers pair designed cox1-F 5’-ACTTCATTGCTTTTGTGTTTATA-3’ and cox1-R 5’-TGCTGTCATAAATGAACCAACAGT-3’ were synthesized by Metabion International AG (Martinsried/Deutschland).

PCR Amplification Protocol
Fragments of the mitochondrial cytochrome c oxidase subunit 1 gene were amplified using designed primers and each PCR mix was prepared in 50 µl total volume with 1 µl of template (50 ng), 10 pMoles of each primer, 45 µl of Ready TaqMix Complete (Mater Mix, AllianceBio, USA), and nuclease free water (Qiagen, Germany) to complete the total volume of the reactions. PCRs were performed in a PTC-100™ Thermal Cycler (MJ Research Inc., USA) using the following cycling protocol: initial denaturation at 95°C for 3 min and then 40 cycles of 94°C for 1 min 50 sec, 58°C for 1 min 30 sec, and 72°C for 1 min. Final extension was carried out at 72°C for 7 min. A reagent blank was run as control in every PCR procedure. Positive results by PCR were retested on two further occasions several days later to examine the reproducibility of PCR. Amplified products from the PCRs were electrophorised on 1.5% agarose gels.
Sequences Analysis

The resulting aligned output was manually adjusted (Lee et al. 2007). Sequences corresponding to the PCR amplification primers were excluded prior to multiple sequence alignment and phylogenetic analysis. The confirmed sequences were then deposited in the EMBL/GenBank Data Libraries of the NCBI. In order to improve the homology statements out group included the NCBI. In order to improve the homology deposited in the EMBL/GenBank Data Libraries of Sequences Analysis were used for sequencing per isolate. At least two independent PCR products were used for sequencing per isolate.

Phylogeny Construction

The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees and node reliability in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 99 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). Neighbor-Joining and UPGMA methods were used to calculate the evolutionary relationship of the Egyptian isolates with genbank references strains (Saitou and Nei 1987).

3. Results

PCR Products of mt cox1 Gene

PCR products were amplified from mt genomes using synthesized primers set. Across the alignment of mt genomes few regions were suitably conserved to allow primer design. The PCR products were approximately 700 bp, 702 bp and 715 bp for mt cox1 gene of H. nana rat isolates, H. diminuta rat isolates, and H. nana human isolates, respectively, (Figure 1).

Sequences Analysis

Variation occurred in terms of sequence length and nucleotide differences and gaps (nucleotide insertions, deletions, and substitutions), but not G+C percentage where the overall numbers did not differ between amplified fragments; A (23%), C (10%), G (22%) and T (45%). Where Nucleotide alterations were found to be variable and several nucleotide insertions, deletions and substitutions were detected with gaps in the same or different positions (Figure 2).

Phylogeny Construction

Similar topologies were observed in the Egyptian isolates with genbank references strains. Optimal phylogenetic tree with the sum of branch length = 1.91459848 is shown (Figure 3). Egyptian species were genetically distinct from other species used in this study that are phylogenetically relating to Hymenolipidid. In addition, despite their host susceptibility differences they all gathered in one cluster with 100% similarity, which was non significantly decreased on internal nodes. Moreover, obviously far away from H. diminuta published sequence AB033412.1 (gi|1399136) who's assumed to be quite genetically closely related to Egyptian H. diminuta than all other H. nana isolates.
Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin.

GenBank accession numbers of Egyptian amplicons were then deposited in the GenBank of NCBI under the following accession numbers; *H. diminuta* (GU433102), *H. nana* rat isolate (GU433103), and *H. nana* human isolate (GU433104).

Fig. 1 PCR products of *mt cox1* gene amplified by specified primers pair from Egyptian isolates of (A) *Hymenolipis diminuta* 703 bp fragment, (B) *H. nana* rat isolate 699 bp fragment, (C) *H. nana* human isolate 715 bp fragment, and (M) 100 bp DNA Ladder.

4. Discussion: Mitochondria play a central role in metabolism, apoptosis, disease, and aging (Le et al. 2002). They are the site of oxidative phosphorylation, essential for the production of ATP, as well as a variety of other biochemical functions. Within these subcellular organelles is a genome, separate from the nuclear chromatin, referred to as mitochondrial DNA (mtDNA), very commonly used in studies of molecular phylogenetics (Avise 2000). Flatworm mitochondrial genomes have a number of distinct features including all genes are coded on the same strand (von Nickisch-Rosenegk et al. 2001), utilize a unique mitochondrial genetic code (Boore 1999) and truncated stop codons have also been found among a number of genes (Nakao et al. 2000, 2003). In the current study the earliest genbank records of cox1 gene of Egyptian Hymenolepis spp. are declared. So far, only a few is known about the relative divergence rates of mitochondrial DNA in *hymenolipis species* especially Egyptian isolates (Vilas et al. 2005), hence only one species belonging to *H. diminuta* is completely sequenced and published in genbank (Littlewood et al. 2008). However, PCR technology and DNA sequencing techniques permit the identification of species, strains, and populations from any stage in their life history to distinguish among morphologically similar parasites (Boore 1999).

Egyptian species were genetically distinct from other species used in this study that are phylogenetically relating to Hymenolipidid. Both Egyptian murine isolates of Hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin. WHO annual reports maintained the traditional host specificity of hymenolipidid till few years ago (Lee et al. 2007). Unfortunately, the unexpected discovery of a mixed infection with specie which is known to infect rodent as definitive host in surveyed individuals (Thompson et al. 2001; Macnish et al. 2003) as well as in dogs living in the same locality as their infected owners declared the public health impact of new infections, and meditating urgent thorough understanding of the epidemiology of these parasites (Jenkins and Andrew 1993; Thompson et al. 1993; Macnish et al. 2003). Since such deviation in patency of infection was not previously recorded thus highlights the growing importance of using molecular techniques in both the detection and characterization of parasite species in human and animals' populations especially between morphologically similar species (Okamoto et al. 1997; Nakao et al. 2000; von Nickisch-Rosenegk et al. 2001; Macnish et al. 2003).

In a comparison of genetic makeup, our result suggests that cox1 gene is generally conserved by each isolate nucleotide sequence analysis. Despite of variation occurred in terms of sequence length and nucleotide differences and gaps (nucleotide insertions, deletions, and substitutions), but not G+C percentage where the overall numbers did not differ between amplified fragments mostly triggered by a variety of hosts' biological conditions (Macnish et al. 2002a, b). Such data showed consistent patterns with other researcher groups in this regard. They reported that mitochondrial DNA sequences of the Platyhelminthes accumulate nucleotide substitutions at a much higher rate than sequences in comparisons.
of genetic distances (Littlewood and Bray 2001; Vilas et al. 2005). Base substitutions and additions are characterized by high T content which can, at times, represent poly-T structures. In addition, this may be a consequence of frame-shift mutations or premature stop codons, however, protein-coding genes of the mtDNA are error-checked by translating the nucleotide sequences (Benasson et al. 2001). Specific substitution rates include metabolic rates and body mass, generation time, differential fixation of slightly deleterious mutations, DNA repair mechanisms, and nucleotide composition (Vilas et al. 2005).

According to the inferred topology of amino acids phylogeny of Egyptian hymenolipid, hosts effect on the evolutionary relationship between isolates was clear despite their intra species differences which agree with Johnston (2006). This could explain the closer relation of Hymenolipis spp. (H. nana and H. diminuta) collected from rat to be arranged in one cluster despite the disparities in host species and morphology which is in continence with Littlewood et al. (2008). These results agree with previous reports supported variant biological features of H. diminuta that are not always identical between isolates is built on genetic background (Okamoto et al. 1997). However, there results are conflicting with both the characteristic cryptic species of H. nana (Macnish et al. 2002a, b), and Schmidt classification where H. nana should be closer to H. microstoma than H. diminuta (Schmidt 1986). These observations that were revealed from the present study which should not be applied unambiguously to host-parasite associations since it does not take into consideration other factors related to the ecology of the hosts and the dynamics of the host-parasite assemblages (Johnston 2006). However, it should highlight the danger of triggering changes in genetic interspecificity subsequently definitive host susceptibility. Since, mt cox1 resultant phylogenetic tree did not support the current hypotheses on the basis of morphological evidence for the separation of species (Littlewood et al. 2008).

In conclusion, molecular protocol developed in this study will provide the tools for achieving supplementary comprehensive epidemiological portrait of infection in Egypt. Consequently, should be applied on much broader scale in screening for Hymenolipis spp. infections. Sufficient clarification of evolutionary relationship of Hymenolipis spp. by other ribosomal DNA content, and complete mt genome sequencing and its genes arrangement are essentials. These data will ultimately aid investigations on dynamics of morphological and developmental evolution, as well as the biology of parasitism.

Corresponding author
Omnia M. Kandil
Parasitology and Animal Diseases Department, National Research Center, Dokki, Giza, Egypt, kandil_om@yahoo.com*

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