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

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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.

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1. Introduction:

Hymenolepis 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 Hänel 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 (*mt*DNA) have been proven useful in biology, epidemiology and diagnosis of several parasitic infections of human and veterinary importance (Ngarmamonpirat 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 *mt*DNA 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 *Hymenolopis* 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 *mt*DNA 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 approximately 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 ^{o}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 QIAmp 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`-ACTTCATTGCTTTTGCTTTTTAGA-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^{0}C for 3 min and then 40 cycles of 94^{0}C for 1 min 50 sec, 58^{0}C for 1 min 30 sec, and 72^{0}C for 1 min. Final extension was carried out at 72^{0}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

(Bioshop Canada, Burlington, Ontario, Canada) stained with ethidium bromide (0.5 µg/ml) (Bioshop Canada) (Sambrook et al. 1989). A 100 bp ladder (Jena Bioscince, GmbH, Germany) was loaded in each gel then photographed under UV light with gel documentation system.

Sequencing of *cox1* Gene Products

PCR-product of each isolates were purified with QIAquick-spin PCR purification kit (Qiagen, Germany) then directly sequenced from both directions using ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem, FosterCity, California) according to manufacturer's instructions on a 3130XL Genetic Analyzer (Applied Biosystems). At least two independent PCR products were used for sequencing per isolate.

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 *Taenia saginata* (AB465239.1), *T. solium* (AY211880.1), *T. multiceps* (GQ228818.1), *Echinococcus granulosus* (AF314223.1), *E. multilocularis* (AF314223.1) and *Spirometra erinaceieuropaei* (AB374543.1), as well as all annotated sequences of *Hymenolepis diminuta* (AB033412.1) and *Hymenolepis nana* (AF314223.1, AY121842.1, AB494471.1, AB494472.1, AB033412.1, AF314223.1) by Basic Local Alignment Search Tool (nBLAST) (www.ncbi.nih.gov/BLAST/) in the NCBI database (National Center for Biotechnology Information, NIH, Bethesda, Maryland, USA) (Tatusova and Madden 1999). The alignment gaps were treated as missing data. Phylogeny of Egyptian *Hymenolipis nana* and *H. diminuta* human and rat isolates based on *coxI* gene partial sequences and multiple alignment analysis were performed with CLUSTAL W computer program (Thompson e al. 1994).

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 (Zuckerkand 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 three genbank published isolates of *H. nana*; AB033412.1 (gi|6045204), AB494472.1 (gi|2262378), and AY121842.1 (gi|2221354), forming one clade 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

The annotated sequences of Egyptian isolates 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).

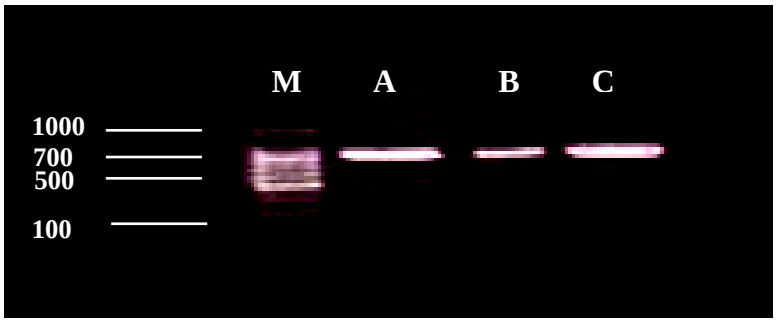

Fig. 1 PCR products of *mt cox1* gene amplified by specified primers pair from Egyptian isolates of (A) *Hymenolipis diminuta* 703 bp fragment, (B) *H. nana* rat isolate 699 bp fragment, (C) *H. nana* human isolate 715 bp fragment, and (M) 100 bp DNA Ladder.

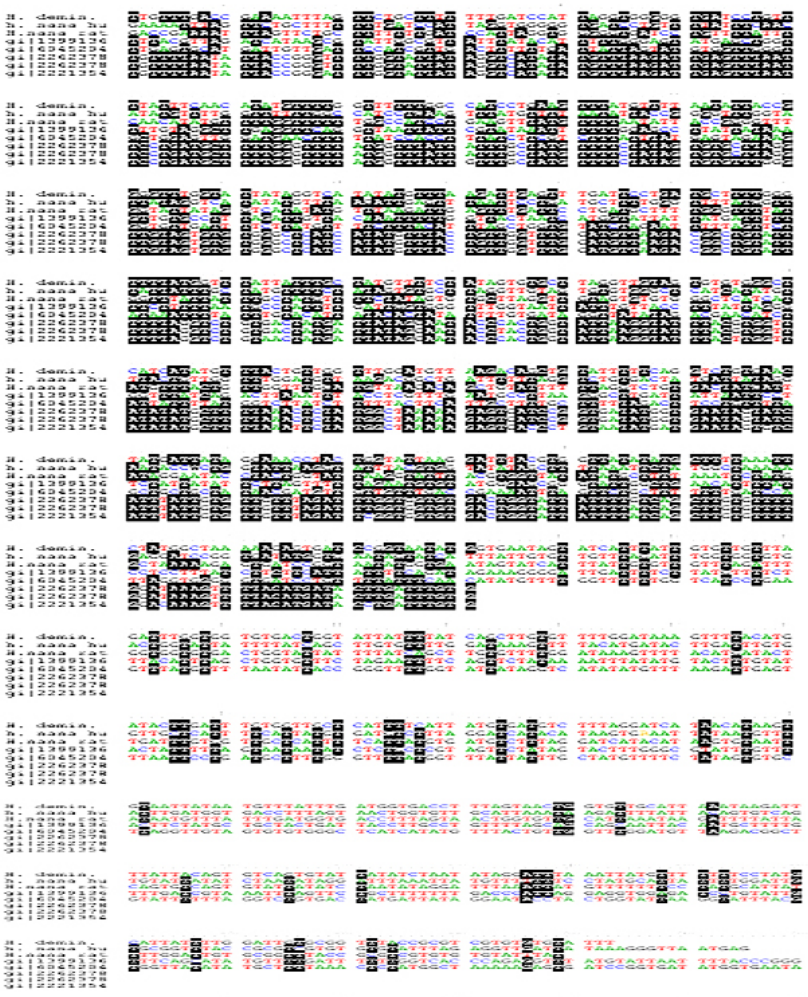

Fig. 2 Nucleotides multiple alignment of partial *mt cox1* gene sequences of Egyptian *H. diminuta*, *H. nana* human isolate, *H. nana* rat isolate, and reference gi|13991366: *H. diminuta*, gi|226237884: *H. nana* isolat: HnanaMon, gi|22213549:*H. nana*, gi|6045204: *H. nana*, gi|14009612: *Echinococcus granulosus* genotype 1, gi|15042575: *Echinococcus equinus* , gi|193884329: *Spirometra erinaceieuropaei*, gi|239997751: *Taenia multiceps*, gi|28856111: *Taenia solium*, and gi|260162222: *Taenia saginata*, isolate: TsagT017KANTH. Black columns represent identical nucleotide sequences between aligned isolates.

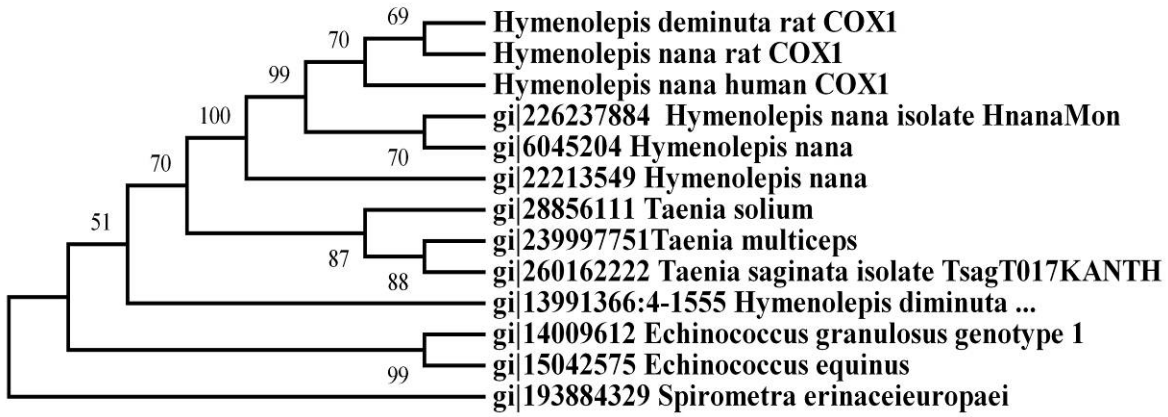

Fig. 3 Rooted phylogenetic tree based on amino acids sequences of in silico translated partial *mt cox1* gene sequences of Egyptian *H. diminuta, H. nana* human isolate, *H. nana* rat isolate and reference gi|13991366: *H. diminuta*, gi|226237884: *H. nana* isolate: HnanaMon, gi|22213549: *H. nana*, gi|6045204: *H. nana*, gi|14009612: *Echinococcus granulosus* genotype 1, gi|15042575: *Echinococcus equinus*, gi|193884329: *Spirometra erinaceieuropaei*, gi|239997751: *Taenia multiceps*, gi|28856111: *Taenia solium*, and gi|260162222: *Taenia saginata*, isolate: TsagT017KANTH. Similar topologies were developed when both Neighbor-Joining and UPGMA methods were applied (MEGA4 software).

4. Discussion:

Mitochonderia 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 hymenolepidid 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 *mt*DNA 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 hymenolipidid, 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.

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