Abstract

*Hymenolepis nana*, typically a parasite found in conventionally established mouse colonies, has zoonotic potential characterized by auto-infection and direct life cycle. The objective of this study was to determine the rate of parasite infection in laboratory mice. The hymenolepidide cestode infected 40% of the 50 mice sampled. The rate of infection in males (52%) was higher than in females (28%). Morphological studies on the cestode parasite showed that worms had a globular scolex with four suckers, a retractable rostellum with 20–30 hooks, and a short unsegmented neck. In addition, the remaining strobila consisted of immature, mature, and gravid proglottids, irregularly alternating (Pakdel et al., 2013), and also known to harbor several ecto-ectosystems, acting as prey, or carriers of disease and reservoirs of infections linked to increased human zoonotic diseases (Kataranovski et al., 2010). Increased rodent populations in an area could be directly linked to increased human zoonotic diseases (Khatoon et al., 2004). The parasite taxonomy was confirmed by the analysis of the mitochondrial cytochrome c oxidase subunit 1 (mtCOXI) gene. The parasite recovered was up to 80% identical to other species in GenBank. High blast scores and low divergence were noted between the isolated parasite and previously described *H. nana* (gb| AP017666.1). The phylogenetic analysis using the COXI sequence places this hymenolepidid species of the order Cyclophyllidea.

**Key words:** Hymenolepis species, laboratory animals, molecular analyses, morphological characterization, rodents

Introduction

Laboratory animal models are widely used in biological experiments (Perec-Matysiak et al., 2006). The most common of them used in different research fields are rodents as mice and rats (Mehlhorn et al., 2005). They are a vital component of different cultural fields, stored grains, and various edible commodities in houses, resulting in disease spread (Khatooon et al., 2004). The ability of rodents to act as vectors is significantly increased, owing to their physiological similarities with humans (Kataranovski et al., 2010). Increased rodent populations in an area could be directly linked to increased human zoonotic diseases (Stojcevic et al., 2004).

Morphological, Molecular, and Pathological Appraisal of *Hymenolepis nana* (Hymenolepididae) Infecting Laboratory Mice (*Mus musculus*)

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**Micrographia**

Hymenolepididae Ariola, 1899 is a diverse family of cyclophyllidean tapeworms that infects approximately 620 bird species and 230 mammal species (Czapinski & Vaucher, 1994). *Hymenolepis Weinland, 1858* is a genus characterized by having an unarmed scolex and a rudimentary rostellar apparatus. It is mainly a parasite in rodents; a few species in bats and one in hedgehogs have been reported. Members of this genus have been reported in Africa, Asia, Paleartic, Nearctic, Ethiopia, and Oriental regions (Thompson, 2015). Rodents are the main definitive hosts of both *Hymenolepis nana* and *H. diminuta*, which are zoonotic and known as the dwarf and rat tapeworms, respectively (Steinmann et al., 2012). *H. nana* is the most common cestode infecting humans, whereas *H. diminuta* causes occasional human infections (Soares Magalhães et al., 2013). *H. nana* is the only cestode capable of completing the life cycle in the final host without the need for an intermediate host. Infection is most commonly acquired from eggs in an infected individual’s feces, which spread by contaminated food (Smyth & McManus, 1989). Infections with *H. nana* in the primary stage are often asymptomatic. Nevertheless, as the disease progresses to the chronic stage, the host manifests symptoms as diarrhea, abdominal pain, nausea, and dizziness (Huda-Thaher, 2012). *H. nana* infections linked to low intestinal vitamin B12 absorption (Mohammad & Hegazi, 2007). Hymenolepiasis diagnosis and causative species differentiation require the analysis of the eggs recovered from the host feces to
identify morphological characteristics (Nkouawa et al., 2016). Advanced molecular biology including techniques, such as polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), are simple and rapid methods for parasite identification (Perec-Matysiak et al., 2006; Robles & Navone, 2007). In particular, PCR-RFLP is commonly used to identify and classify helminth parasites accurately including cestodes (Francisco et al., 2010; Rokni et al., 2010; Mahami-Oskouei et al., 2011; Teodoro et al., 2011). However, the phylogenetic relationships of Hymenolepididae at the family and generic levels remain elusive (Czaplinski & Vaucher, 1994). Hymenolepis species’ taxonomic status and systematics are problematic, primarily because of the presence of cryptic species (Haukisalmi et al., 2010). The current, nuclear rDNA internal transcribed spacer (rDNA-ITS1 and ITS2) sequence data are considered to have the revolutionized phylogenetic analysis as a powerful tool for resolving remarkable taxonomic issues and discriminating closely related genera and species (Coleman, 2003). In addition, the use of rDNA-ITS2 to predict secondary structures from primary sequence data may provide additional information for species identification at a higher taxonomic level (Schultz et al., 2005; Ghatani et al., 2012). The mitochondrial cytochrome c oxidase subunit 1 (mtCOX1) marker has also been used successfully at family and genus levels to infer and establish phylogenetic Cyclophyllidea relationships (Sharma et al., 2016).

In this study, natural prevalence and morphological as well as molecular characteristics of the partial mtCOX1 genes of *H. nana* species infecting laboratory mice (*Mus musculus*) were evaluated to determine the exact taxonomic and phylogenetic position of this parasite species. In addition, the study examined the impact of sex differences on the prevalence of parasite infection and the role of laboratory mice as reservoirs of hymenolepidid tapeworms.

**Materials and Methods**

*Experimental Animal Collection*

A total of 50 adult male and female laboratory mice *Mus musculus* (family: Muridae) were randomly selected from the Laboratory of Animal Breeding Council (King Saud University of Medical Science, Riyadh, Saudi Arabia). They were housed under controlled temperature (24 ± 2°C), light (12 h light/dark cycle), and relative humidity (40–70%) in a room. A standard diet and water *ad libitum* were given to them. The mice were anesthetized and killed by placing them in a small container with ether in accordance with the ethical standards for handling of experimental animals recommended by the King Saud University Ethics Committee, Riyadh, Saudi Arabia. The animals were tested for any external signs of infection. After dissection, the internal organs were removed and examined for worm infections.

*Parasitological Examination*

**Light Microscopic Studies**

The recovered cestode parasites were placed in saline solution, fixed in warm alcohol–formalin–acetic acid solution, preserved in 70% alcohol, stained with Semichon’s acetocarmine, dehydrated in ascending grades of alcohol, cleared in clove oil, and then mounted in Canada balsam. With the aid of Yamaguti’s identification key (1959), the worms were identified. Parasite prevalence was calculated according to the formula of Bush et al. (1997). Adult specimens were examined and photographed using a microscope Leica DM 2500 (NIS ELEMENTS software, v. 3.8). Measurements are recorded in millimeters and shown as the range followed by mean ± standard deviation in parentheses.

**Scanning Electron Microscopic Studies**

Specimens were fixed in 3% glutaraldehyde, washed with a buffer of sodium cacodylate, dehydrated in a graded ethanol series, and infiltrated with amyl acetate. They were then passed through an ascending series of Geneosolv D, processed in a critical point dryer (LEICA EM CPD300) with Freon 13, and then coated with gold–palladium using an auto-fine coater (JEOL, JEC-3000FC). The samples were then analyzed and photographed at 10 kV in a JEOL scanning electron microscope (JSM-6060LV) at the Central Laboratory, King Saud University, Riyadh, Saudi Arabia.

**Histopathological Examination**

The mouse intestines were collected and fixed for 24 h in 10% neutral formalin immediately after mice sacrifice, and paraffin blocks were generated and routinely processed for light microscopy. The resulting sections of 4–5 μm were stained with hematoxylin and eosin and then visualized to evaluate pathological changes using a microscope Leica DM 2500 (NIS ELEMENTS software, v. 3.8).

**Molecular Analyses**

Genomic DNA was extracted using a QIAamp DNA mini Kit (Qiagen, Venlo, Netherlands) from ethanol-preserved samples as recommended by the manufacturer. A partial gene region of mtCOX1 was amplified using primers designed by Nkouawa et al. (2016), including Hym-cox1F (5′-GGT ACT AAT CAT GGT ATT ATT ATG-3′) and Hym-cox1R (5′-CCA AAA TAA TGC ATA GGA AAA-3′). Amplicons were sequenced using a 310 automated DNA sequencer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with the help of an ABI Prism Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA). A BLAST search was performed to identify related sequences from the NCBI database. The mtCOX1 gene sequences were aligned using the CLUSTAL-X multiple sequence alignment (Thompson et al., 1997). A phylogenetic tree with maximum parsimony [neighbor-interchange (CNI) level 3, random addition trees = 100] was built using MEGA v. 6.0. The bootstrap analysis was conducted to determine the robustness of the tree topologies based on 1,000 replicates.

**Results**

Of the 50 mice hosts, 20 (40%) were infected naturally. The rate of infection in males (52%; 13/25) was higher than that in females (28%; 7/25). A total of 243 specimens of Hymenolepididae species were recovered from the laboratory mice’s small intestines.

**Microscopic Examination**

The strobila length was 3.74 ± 0.1 (2.53–5.70) mm, with a maximum width at pre gravid or gravid proglottids, 0.094 ± 0.02 (0.016–0.270) mm (Figs. 1, 2, Table 1). There were distinct metamerisms, craspedote, serrate margins, and proglottids which were wider than long. The scolex globular length was 0.125 ± 0.01 (0.113–
0.164 mm, with a maximum width of 0.287 ± 0.01 (0.221–0.295) mm at four suckers and a retractable rostellum. The rostellum, located at the scolex center, 0.027 ± 0.001 (0.022–0.047) mm in length by 0.065 ± 0.001 (0.047–0.081) mm in width, had an irregular surface without microtriches, armed with 20–30 hooks, which were also retractable into a contractile rostellar pouch measuring 0.099 ± 0.002 (0.089–0.125) mm in length by 0.091 ± 0.01 (0.083–0.137) mm in width. The diameter of each hooklet was 0.35 ± 0.01 (0.29–0.42) mm. Suckers were rounded or oval in shape, unarmed, and 0.095 ± 0.002 (0.081–0.167) mm in length by 0.084 ± 0.002 (0.062–0.104) mm in width. The scolex was approximately 0.157 ± 0.01 (0.156–0.234) mm in diameter, followed by a short unsegmented neck region.

There were two pairs of longitudinal canals in the excretory system. Each pair was 0.098–0.127 mm from the lateral proglottid margins. Transverse anastomoses connected the ventral osmoregulatory canals, while the dorsal ones moved bilaterally to the lateral proglottid margins in relation to the ventral canals. Proglottid development was progressive and protandrous, with external segmentation being evident at the premature strobila section.
Mature proglottids had a length of 0.104 ± 0.05 (0.089–0.157) mm by 0.402 ± 0.09 (0.395–0.563) mm in width. Genital pores were unilateral, irregularly alternating, and slightly located anterior to the middle of each proglottid. The genital ducts passed dorsally to the longitudinal osmoregulatory canals, both ventral and dorsal.

Three sub-spherical testes arranged in a transverse row, one poral and two aporal, but not in contact with the longitudinal excretory canals, and 0.068 ± 0.002 (0.047–0.098) mm in length by 0.071 ± 0.001 (0.066–0.102) mm in width. The vas deferens expanded to form an external seminal vesicle of 0.109 ± 0.02 (0.089–0.174) mm in length by 0.062 ± 0.003 (0.045–0.088) mm in width. The cylindrical cirrus sac was 0.054 ± 0.001 (0.042–0.087) mm in length by 0.143 ± 0.04 (0.091–0.280) mm in width and did not extend beyond the longitudinal excretory canals. The internal seminal vesicle was 0.040 ± 0.001 (0.031–0.078) mm in length by 0.086 ± 0.003 (0.068–0.165) mm in width and occupied almost the entire cirrus sac. The slightly elongated external seminal vesicle, 0.073 ± 0.001 (0.060–0.079) mm in length by 0.039 ± 0.001 (0.031–0.042) mm in width, was located at the anterior half of the proglottids.

Initially, after the cirrus sac, the vagina gradually expanded into the voluminous seminal receptacle, measuring 0.201 ± 0.03 (0.185–0.298) mm in length by 0.017 ± 0.001 (0.010–0.020) mm in width.
Table 1. Main morphological features and measurements of *H. nana* compared with those in previous studies.

| Related species | Host species (locality) | Body size | Scolex | Suckers | Rostellum | No. of rostellar hooks | Testes | Ovary | Vitellarium | Eggs | Embryophore | Embryonic hook |
|-----------------|-------------------------|-----------|--------|---------|-----------|----------------------|--------|-------|-------------|------|-------------|---------------|
| *H. asymmetrica* Janičík (1904) | *Microtus arvalis* (Laposák) | 5.0–13.5 × 0.2–0.4 | 0.220–0.300 | 0.085–0.120 | – | 18–21 | – | 0.200–0.400 | – | 0.045–0.067 | 0.020–0.030 | 0.0085–0.009 |
| *H. microstoma* Joyeux & Kobozieff (1928) | *Musculus* (South-Oran) | 3.50 × 0.20 | 0.200 | – | 0.100 | 27 | 0.120 × 0.170 | – | – | 0.080 × 0.090 | 0.030 | 0.017 × 0.020 |
| *H. christensoni* Macy (1931) | *Myotis yumanensis* (Alaska) | 5.4–6.5 × 0.295–0.323 | 0.340–0.434 | 0.104–0.116 | 0.100 | 40 | 0.125 (0.104–0.144) × 0.105 (0.080–0.120) | 0.247 (0.060–0.116) × 0.092 (0.220–0.288) | 0.099 (0.084–0.132) × 0.069 (0.056–0.084) | 0.038 (0.035–0.042) × 0.034 (0.030–0.037) | 0.025–0.032 | 0.020–0.025 |
| *H. citelli* McLeod (1933) | *Citellus tridecemlineatus* (Canada) | 15 × 0.28 | – | 0.113 × 0.245 | 0.038 | Unarmed | – | – | – | 0.078–0.086 × 0.059–0.065 | – | – |
| *H. nagatyi* Hilmy (1936) | *Crocidura occidentalis* (Rutshuru) | 2.5 × 0.810 | 0.467–0.548 | 0.114–0.125 | 0.195–0.225 | 100–110 | – | – | – | 0.040–0.043 | 0.023 |
| *H. roudabushi* Macy & Rausch (1946) | *Eptesicus fuscus* (Iowa) | 3.9–7.4 × 0.270–0.488 | 0.240–0.325 | 0.084–0.096 | 0.120 | 45 | 0.138 (0.092–0.140) × 0.122 (0.112–0.164) | 0.094 (0.080–0.120) × 0.246 (0.180–0.328) | 0.056 (0.036–0.072) × 0.085 (0.068–0.120) | 0.041 (0.035–0.045) × 0.036 (0.032–0.045) | 0.025–0.030 | 0.022–0.30 |
| *H. gertschi* Macy (1947) | *Eptesicus fuscus* (Iowa) | 6.1 × 0.9 | 0.400 | – | 0.031–0.035 × 0.096–0.108 | 50 | 0.084 (0.072–0.100) × 0.076 (0.06–0.088) | 0.099 (0.072–0.125) × 0.163 (0.145–0.180) | 0.067 (0.055–0.092) × 0.090 (0.075–0.117) | 0.052–0.62 | 0.055 | 0.025–0.020 |
| *H. diminuta* Wardle & McLeod (1952) | *Ratus ratorus* (Cosmopolita) | 2.00–3.00 × 0.30–0.40 | 0.299–0.300 | 0.10–0.120 | Rudiment | Unarmed | – | – | – | 0.060–0.070 | – | – |
| *H. lasionycteridis* Rausch (1975) | *Lasionycteris noctivagans* (Ohio) | 6.1 × 0.615–0.734 | 0.168–0.220 | 0.070–0.084 | 0.130 | 38–40 | 0.091 (0.068–0.108) × 0.129 (0.100–0.176) | 0.080 (0.064–0.092) × 0.328 (0.240–0.468) | 0.044 (0.036–0.052) × 0.139 (0.124–0.160) | 0.044 (0.037–0.048) × 0.034 (0.030–0.037) | 0.025–0.032 | 0.013–0.015 |
| *H. geomydis* Gardner & Schmidt (1987) | *Geomyys bursarius* (Colorado) | 72.26–168.41 × 0.198–0.330 | 0.189–0.252 × 0.194–0.245 | 0.092–0.124 × 0.065–0.094 | Rudiment | Unarmed | – | – | – | 0.060–0.070 | – | – |
| *H. rhinopomae* Sawada & Mohammad (1989) | *Rhinopoma microphyllum* (Iraq) | 2.6–2.9 × 0.11–0.12 | 0.280–0.385 × 0.190–0.210 | 0.098–0.112 × 0.091–0.105 | 0.119–0.140 × 0.063–0.084 | Unarmed | 0.056–0.70 × 0.028–0.042 | 0.077 | 0.049–0.063 × 0.028–0.035 | 0.049–0.063 × 0.028–0.035 | 0.032–0.046 | 0.018 |
| *H. dytocodontis* Sawada & Harada (1990) | *Dymecodon pilosistris* (Japan) | 5.6–7.8 × 0.19–0.21 | 0.245–0.280 × 0.231–0.315 | 0.105–0.119 × 0.119–0.154 | 0.140 × 0.056–0.077 | Unarmed | 0.091–0.098 × 0.063–0.070 | 0.280–0.315 | 0.105–0.126 × 0.077–0.084 | 0.064–0.077 × 0.063–0.070 | 0.039 × 0.035 | 0.014 |

(Continued)
| Related species | Host species (locality) | Body size (mm) | Scolex (mm) | Suckers (mm) | Rostellum (mm) | No. of rostellar hooks | Testes (mm) | Ovary (mm) | Vitellarium (mm) | Eggs (mm) | Embryophore (mm) | Embryonic hook (mm) |
|-----------------|------------------------|----------------|-------------|-------------|---------------|----------------------|-------------|------------|-----------------|-----------|-----------------|---------------------|
| *H. magna*      | Rhinolophus affinis (China) | 4.9 (4.3–5.0) × 0.23 (0.22–0.34) | 0.330 (0.310–0.330) | 0.120 (0.116–0.130) × 0.140 (0.132–0.143) | Absent | Unarmed | 0.153–0.176 × 0.116–0.163 | 0.109–0.120 × 0.068–0.087 | – | 0.040–0.41 × 0.041–0.042 | 0.022–0.028 × 0.025–0.30 | 0.014–0.015 |
| *H. microstoma* | Mus musculus (London) | 4.7 (2.5–8.1) × 0.065 (0.029–0.108) | 0.138 (0.116–0.157) × 0.232 (0.204–0.284) | 0.102 (0.079–0.129) × 0.096 (0.076–0.113) | 0.038 (0.026–0.052) × 0.071 (0.051–0.075) | 25 (22–26) | 0.072 (0.051–0.114) × 0.078 (0.061–0.115) | 0.063 (0.034–0.103) × 0.234 (0.130–0.360) | 0.041 (0.038–0.094) × 0.056 (0.032–0.068) | – | – | – |
| *H. asketus*    | Blarina brevicauda (Nebraska) | 1.2–2.0 × – | 0.136–0.142 × 0.052–0.067 × 0.035–0.052 | 0.040–0.075 × 0.029–0.061 | 10 | – | – | – | – | – | – |
| *H. bicauda*    | Apomys microdon (Philippines) | 2.6–2.9 × 0.99–1.19 | 0.260–0.288 × 0.092–0.103 × 0.080–0.095 | – | Unarmed | 0.070–0.103 × 0.065–0.100 | 0.108–0.140 | 0.038–0.055 × 0.050–0.065 | 0.046–0.054 × 0.050–0.060 | 0.027–0.033 × 0.031–0.038 | 0.175–0.19 |
| *H. haukisalmii* | Bullimus luzonicus (Philippines) | 13.2 × 0.24 | 0.240–0.265 × 0.083–0.105 × 0.081–0.093 | – | Unarmed | 0.116–0.160 × 0.085–0.157 | 0.193–0.208 | 0.061–0.083 × 0.080–0.125 | 0.029–0.034 × 0.037–0.046 | 0.015–0.017 × 0.018–0.020 | 0.011–0.013 |
| *H. alterna*    | Rattus everetti (Philippines) | 16.5–17.0 × 0.29–0.38 | 0.380–0.410 × 0.154–0.189 × 0.130–0.144 | – | Unarmed | 0.072–0.111 × 0.065–0.091 | 0.506–0.525 | 0.090–0.163 × 0.125–0.205 | 0.048–0.051 × 0.049–0.053 | 0.023–0.026 × 0.025–0.027 | 0.0123–0.014 |
| *H. bilaterola* | Apomys datea (Philippines) | 8.6 × 0.15–0.25 | 0.347–0.400 × 0.110–0.150 × 0.105–0.120 | – | Unarmed | 0.092–0.126 × 0.075–0.106 | 0.190–0.230 | 0.070–0.085 × 0.080–0.115 | 0.067–0.090 × 0.071–0.103 | 0.035–0.045 × 0.037–0.048 | 0.017–0.0191 |
| *H. diminuta*   | Rattus everetti (Khalakdzonot) | – | 0.146–0.217 × 0.090–0.097 × 0.078–0.090 | – | Unarmed | – | – | – | 0.058–0.073 × 0.045–0.063 | – | – |
| *H. nana*       | Mus musculus (Saudi Arabia) | 3.74 ± 0.1 (2.53–5.70) × 0.094 ± 0.01 (0.016–0.270) | 0.125 ± 0.01 (0.113–0.164) × 0.287 ± 0.03 (0.221–0.295) | 0.095 ± 0.001 (0.081–0.167) × 0.084 ± 0.001 (0.062–0.104) | 0.027 ± 0.001 (0.022–0.047) × 0.065 ± 0.001 (0.047–0.081) | 25 (20–30) | 0.068 ± 0.001 (0.047–0.098) × 0.071 ± 0.001 (0.066–0.102) | 0.061 ± 0.001 (0.029–0.112) × 0.214 ± 0.031 (0.147–0.302) | 0.028 ± 0.001 (0.021–0.088) × 0.050 ± 0.001 (0.047–0.053) | 0.049 ± 0.01 (0.042–0.052) × 0.050 ± 0.001 (0.029–0.037) | 0.012 ± 0.001 (0.010–0.014) × 0.016 ± 0.001 (0.015–0.017) | 0.012 ± 0.001 (0.010–0.014) × 0.016 ± 0.001 (0.015–0.017) |
in width. The ovaries were lobulated, 0.061 ± 0.002 (0.029–0.112) mm in length by 0.214 ± 0.01 (0.147–0.302) mm in width. The vitelline gland compact, measuring 0.028 ± 0.001 (0.021–0.088) mm in length by 0.051 ± 0.001 (0.030–0.063) mm in width, was situated posterior to the ovaries. The average length of the seminal receptacle was 0.047 ± 0.001 (0.035–0.087) mm in length by 0.090 ± 0.002 (0.069–0.170) mm in width. The uterus formed as a transversely elongated perforated sac, located dorsally to other organs, and extending laterally beyond the longitudinal osmoregulatory canals. The uterus formed numerous diverticula on the dorsal and ventral sides during the proglottid development. Testes have been shown to persist in mature proglottids, while in gravid proglottids, the cirrus sac and vagina persist. Gravid proglottids measured 0.154 ± 0.04 (0.123–0.243) mm in length by 0.975 ± 0.16 (0.854–1.021) mm in width. A full developed uterus which occupied the entire midpoint and expanded laterally beyond the longitudinal osmoregulatory canals was saccate and had several ventral and dorsal diverticula; the lateral sides of a gravid uterus were usually not perforated. There were many (up to 200) small eggs in the uterus.

Eggs, 0.049 ± 0.01 (0.042–0.052) mm in length and 0.050 ± 0.001 (0.047–0.053) mm in width, were oval or spherical with a thin hyaline shell. The shell enclosed the embryophore, approximately 0.028 ± 0.001 (0.023–0.030) mm in length by 0.032 ± 0.001 (0.029–0.037) mm in width, with three polar filaments, oncospheres 0.013 ± 0.001 (0.011–0.014) mm in length by 0.040 ± 0.001 (0.032–0.075) mm in width, and three pairs of embryonic hooks arranged in parallel. The size of each embryonic hook was 0.012 ± 0.001 (0.010–0.014) mm in length by 0.016 ± 0.001 (0.015–0.017) mm in width.

**Developmental Biology of Cysticercoid and Adult H. nana in Mice**

During the infection, the infected mice remained asymptomatic (Figs. 3, 4). After *H. nana* eggs were ingested, infection occurred. Oncospheral embryos hatched in fully developed egg shells, emerged from their shells, and penetrated the liver tissue of mice (day 0), where they developed into cisticercoids within 4 days (day 4), through a sequence of developmental stages during which suckers took their final shape and filled the rostrum with hooks. The suckers appeared initially as somewhat refractive rods. Eventually, in the rest of the scolex, the fully developed rostellar hooks emerged from their shells, and penetrated the liver tissue of mice (day 12).

**Taxonomic Summary**

**Parasite name:** Hymenolepis nana (Family Hymenolepididae

**Host:** Laboratory mice Mus musculus Linnaeus, 1758 (Family: Muridae Illiger, 1811)

**Site of infection:** Small intestine of infected mice

**Type-locality:** Laboratory of Animal Breeding Council of Medical Science, King Saud University, Riyadh, Saudi Arabia

**Prevalence and intensity:** 20 out of 50 (40%) specimens were infected, with a total number of 243 cestodes

**Remarks:** The recovered cestode parasite was compared with those collected from different regions: it most closely resembled *H. nana* previously described by Lapage (1951), Roberts & Janovy (2000), Schantz (2006), Richard (2008), Schmidt et al. (2009), Sadaf et al. (2013), and Kim et al. (2014), since all of them had generic characteristics, except that reported by Mayhew (1925), which indicated that the testes were a compound structure. The parasite is also substantially similar to *H. roudbushi* due to the serrate strobila margins as described by Macy & Rausch (1946). It is also similar to *H. microstoma* described by Joyeux & Kobozieff (1928) because it inhabits the same host species, has the same testes arrangement, as well as lobulated ova-ries with an irregular microtrichevoid surface, differing only in the number of rostellar hooks [20–30 versus 25 (22–26)].

**Molecular Analyses**

A total of 699 bp of the mtCOX1 gene sequence were deposited in GenBank for the cestode parasite being studied (gb|MK133141.1), and the GC content was determined (32%). A unique genetic sequence has been discovered by comparisons of the genomic sequence of the isolated parasite species with different alternative group species and genotypes. The comparison between this novel genetic sequence and others obtained from GenBank showed up to 80% similarity (Table 2). Comparing the nucleotide sequences and divergence, the COX1 gene sequence of this cestode species showed the highest BLAST scores with the lowest divergence values for *H. nana* (gb|AP017666.1, GU433104.1, KT951722.1, LC063187.1, AB033412.1, GU433103.1), *H. diminuta* (gb|GU433102.1, AF314223.1, AP017664.1, KC990401.1), and *H. microstoma* (gb|AP017665.1) (Fig. 5). Analyses involved 29 nucleotide sequences (Fig. 6). In order to estimate the maximum likelihood values, a topology for the tree was automatically computed. The constructed dendrogram revealed two orders in the Eucestoda subclass; Pseudophyllidea and Cyclophyllidea, with the former represented by *Spirometra erinaceieuropaei* (gb|AB015754.1) in the Diphyllobothriidae family, with 80% sequence similarity. Four families represent the latter: Taeniidae, Anoplocephalidae, Mesocestoididae, and Hymenolepididae. Such analyses showed that Taeniidae formed the sister group with strong nodal support for Hymenolepididae. In addition, a clade, including parapylythecian Hymenolepididae, was recovered from the current analysis. Our phylogenetic analysis, which combined new and existing data, showed that the hymenolepid species analyzed was within the Cyclophyllidea order. It has been found that the present species is deeply embedded in the genus *Hymenolepis*, with close relationships to the previously described *H. nana* (gb|AP017666.1), as a putative sister taxon.

**DISCUSSION**

Parasitic laboratory animal infections, even in the absence of clinical signs, can act as an important variable during experimental testing and can potentially infect staff and researchers (Rehbinder et al., 1996; Rosas, 1997; Gonçalves et al., 1998; Bazzano et al., 2002). During experiments, rodent colonies are often contaminated with helminths in conventional animal facilities or may become infected in research laboratories (Rehbinder et al., 1996).

In this study, adult *Hymenolepis* species were recovered from the small intestine of 40% of the laboratory mice, representing a moderate prevalence value. This rate is considerably similar to that reported in the Belgrade area of Serbia (37%; Kataranovski et al., 2010) and the Tarai region of Uttarakhand (40–44%; Sharma et al., 2013). Conversely, it is higher than that reported in Korea (5%), the Philippines (19%; Fedorko, 1999), Grenada
(16%; Coomansingh et al., 2009), Dhaka (27%; Gofur et al., 2010), Addis Ababa (7–27%; Gudissa et al., 2011), the Tabriz of Iran (4%; Garedaghi & Khaki, 2014), the suburban area of Hamadan City of Western Iran (17%; Yoisefi et al., 2014), Italy (29%; D’Ovidio et al., 2015), and the Heilongjiang Province of China (6–15%; Yang et al., 2017). It is also lower than the infection rate reported in Manila (64%; Tubangui, 1931), Japan (53%; Perec-Matysiak et al., 2006), Shiraz of Southern Iran (67%; Tanideh et al., 2010), the City of Aracaju in the Sergipe State of Brazil (67%; Guimarães et al., 2014), and Ahvaz of South-West Iran (63%; Rahdar et al., 2017).

In this study, the prevalence of cestode infections in males was higher than in females, in line with the findings of Folstad & Karter (1992) and Gofur et al. (2010), whom indicated that the typical male bias in parasitism could be due to the evolutionary mechanisms of endocrinological and behavioral sex differences produced to maximize the reproductive production of each sex. Klein (2004) showed that sex differences in the parasite burden are usually attributed to pleiotropic effects of the steroid hormone, especially the possible immunosuppression associated with increased testosterone levels. Hayward (2013) and Hämäläinen et al. (2015) reported that the sexual bias in infection rates could be attributed to different behavioral repertoires, which in turn could be mediated by hormonal conditions. By contrast, Bhuiyan et al. (1996) reported in the following three rodent’s species: Bandicota bengalensis, Rattus rattus, and M. musculus that the prevalence of infection in females was higher than that in males.

Morphological variability associated with cestode taxonomy has received comparatively little attention. Although several
studies have identified variants of character by Mayhew (1925),
and Schiller (1959), few researchers have attempted to determine
their rate of occurrence in the host species. The study of the
Hymenolepididae family members was carried out using samples
from naturally infected hosts. Of the several variant characters
known in hymenolepidid cestodes, (i) the scolex shape and size,
(ii) sucker shape and size, (iii) number, shape, size, and location
of rostellar hooks, (iv) number, size, shape, and relative position
of the testes, (v) irregularities in the number and branching of
the vasa efferentia, (vi) ovary and vitellarium shape, (vii) position
of the genital pores, and (viii) the size of egg structures constitute
important taxonomic characters in this family and have thus been
the most satisfactorily described.

The present Hymenolepis species can be separated from all
comparable species by its smaller scolex size. However, it differs
from other hymenolepidid species, owing to its rudimentary ros-
stellum as that in H. citelli and H. diminuta (Wardle & McLeod,
1952), H. geomydis (Gardner & Schmidt, 1987), H. rhinopoma-
(Sawada & Mohammad, 1989), H. dymecodontis, and H. magna
(Makarikova et al., 2010), H. bicauda, H. haukisalmii, H. alterna,
and H. bilaterala. It also differs from other hymenole-
pidid species, owing to its 20–30 rostellar hooks, as opposed to
18–21 in H. asymmetrica (Janicki, 1904), 40 in H. christensoni
(Macy, 1931), 100–110 in H. nagatyi (Hilmy, 1936), 45 in H. rou-
dabushi, 50 in H. gertschi (Macy, 1947), and 38–40 in H. lasiony-
cteridis (Rausch, 1975). It differs from H. christensoni, owing to its
serrate strobila margins, and from H. asketus (Brooks & Mayes,
2011) because of its 20–30 rather than 10 rostellar hooks, which
are longer (0.089–0.125 versus 0.0013–0.0018), its linear arrange-
ment of testes, and lobulated, as opposed to dumbbell-shaped
ovaries. Our Hymenolepis species also differs from H. asymmetrica,
H. christensoni, H. citelli, H. gertschi, H. lasionycteridis,
H. geomydis, H. dymecodontis, H. magna, H. microstoma,
H. haukisalmii, H. alterna, and H. bilaterala, owing to its smaller
strobila size and the shape of its lobate ovary. Its egg sizes are also
smaller, compared to those of H. citelli (McLeod, 1933), H. gert-
schi (Macy, 1947), H. dymecodontis (Sawada & Harada, 1990),
and H. bilaterala.

The immuno- and developmental biology of H. nana are
unique in mice, due to the parasite’s ability to complete its entire
life cycle; from the egg to the adult worm (direct life cycle) in a
single mouse (Ito, 2015). It occurs when H. nana eggs are ingested
by mice. Ito (2003) and Okamoto (2003) reported that this cycle
can only occur once, as egg-infected mice develop resistance to
reinfection within a few days. Okamoto (2003) reported this
acquired immunity to be thymus dependent. In addition, a single
oncosphere invasion of the intestinal tissue is enough to elicit
rapid immunity from egg reinfection (Baylis, 1924; Joyeux &
Kobozieff, 1928; Ito & Yamamoto, 1976). The subsequent devel-
opment and maturation of parasites depend on eosinophilic infil-
tration and physical pressure that pushes the invaginated juveniles
into the intestinal lumen for adult worms to fully mature; this is
consistent with the findings of Moniez (1880), Nicoll & Minchin
(1910), and Niwa et al. (1998).

Based on their morphology, transmission patterns, or patho-
logical effects on hosts, intestinal cestode parasites are frequently
identified and distinguished. These criteria, however, are often
insufficient to identify specifically (Lichtenfels et al., 1997;
Andrews & Chilton, 1999). Increased knowledge of the genus
Hymenolepis has contributed to the possibility of using molecular
approaches for cestode identification. The mtCOX1 gene was use-
ful in the identification of genetic differences in H. nana that

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**Fig. 4.** Direct life cycle of H. nana inside the laboratory mouse Mus musculus (by own production).
Table 2. Cestoda species used in the phylogenetic analysis of *H. nana* specimens obtained from this study.

| Parasite species                  | Order/family                  | Host/host group (origin)       | Accession no.           | GC content (%) | Percent identity (%) |
|-----------------------------------|-------------------------------|--------------------------------|-------------------------|----------------|-----------------------|
| *Echinococcus granulosus*         | Cyclophyllidea/Taeniidae      | Wild animals (Iran)             | KX269862.1              | 36.2           | 81.0                  |
| *Spirometra erinaceieuropaei*     | Diphyllobothriidea/Diphyllobothriidae | *Homo sapiens* (Japan)           | AB015754.1             | 34.2           | 80.0                  |
| *Moniezia benedeni*              | Cyclophyllidea/Anoplocephalidae | Cattle intestine (Iraq)         | MH259797.1              | 33.9           | 80.0                  |
| *Moniezia expansa*               | Cyclophyllidea/Anoplocephalidae | Sheep intestine (Iraq)          | MH259795.1              | 33.2           | 80.0                  |
| *Paranoplocephala kalelai*       | Cyclophyllidea/Anoplocephalidae | *Microtus voles* (Europe)       | AY189959.1              | 32.6           | 81.0                  |
| *Paranoplocephala omphalodes*    | Cyclophyllidea/Anoplocephalidae | *Microtus voles* (Europe)       | AY189954.1              | 36.7           | 81.0                  |
| *Paranoplocephala* sp.           | Cyclophyllidea/Anoplocephalidae | *Microtus voles* (Europe)       | AY181537.1              | 36.5           | 81.0                  |
| *Mesocestoides litteratus*       | Cyclophyllidea/Mesocestoididae | *Vulpes vulpes* (Central Europe) | JF268525.1          | 30.3           | 80.0                  |
| *Anoplocephala manubriata*       | Cyclophyllidea/Anoplocephalidae | Wild elephant (Sri Lankan)      | KU903287.1             | 31.0           | 81.0                  |
| *Anoplocephala magna*            | Cyclophyllidea/Anoplocephalidae | *Microtus miurus* (Japan)       | AB099691.1             | 32.3           | 81.0                  |
| *Anoplocephala perfoliata*       | Cyclophyllidea/Anoplocephalidae | *Microtus miurus* (Japan)       | AB099690.1             | 33.3           | 81.0                  |
| *Hymenolepis microstoma*         | Cyclophyllidea/Hymenolepididae | *Microtus miurus* (Japan)       | AP017665.1             | 28.0           | 83.0                  |
| *Pseudanoplocephala crawfordi*   | Cyclophyllidea/Hymenolepididae | Unidentified host (China)       | KR611041.1             | 30.3           | 84.0                  |
| *Taenia saginata*                | Cyclophyllidea/Taeniidae      | *Homo sapiens* (Thailand)       | AB533173.1             | 30.9           | 83.0                  |
| *Taenia asiatica*               | Cyclophyllidea/Taeniidae      | *Homo sapiens* (Japan)          | AB608739.1             | 31.1           | 82.0                  |
| *Taenia serialis*                | Cyclophyllidea/Taeniidae      | Wild Rabbits (China)            | KY007158.1             | 30.4           | 83.0                  |
| *Hymenolepis diminuta*           | Cyclophyllidea/Hymenolepididae | *Rattus norvegicus* (Egypt)     | GU433102.1             | 31.7           | 96.0                  |
| *H. nana*                        | Cyclophyllidea/Hymenolepididae | *Rattus norvegicus* (Egypt)     | GU433103.1             | 31.6           | 97.0                  |
| *Rodentolepis fraterna*          | Cyclophyllidea/Hymenolepididae | *Rattus norvegicus* (Spain)     | JN258053.1             | 31.6           | 84.0                  |
| *H. nana*                        | Cyclophyllidea/Hymenolepididae | *Homo sapiens* (Egypt)          | GU433104.1             | 32.0           | 96.0                  |
| *H. nana*                        | Cyclophyllidea/Hymenolepididae | *Mus musculus* (Spain)          | AP017666.1             | 27.1           | 96.0                  |
| *H. nana*                        | Cyclophyllidea/Hymenolepididae | *Homo sapiens* (Japan)          | KT951722.1             | 27.0           | 95.0                  |
| *H. nana*                        | Cyclophyllidea/Hymenolepididae | *Mus musculus* (China)          | LC063187.1             | 31.3           | 95.0                  |
| *H. nana*                        | Cyclophyllidea/Hymenolepididae | *Homo sapiens* (Japan)          | AB033412.1             | 31.9           | 95.0                  |
| *Hymenolepis diminuta*           | Cyclophyllidea/Hymenolepididae | *Rattus rattus* (India)         | AF314223.1             | 29.0           | 82.0                  |
| *Hymenolepis diminuta*           | Cyclophyllidea/Hymenolepididae | *Tribolium castaneum* (Unidentified origin) | KC900401.1 | 31.3           | 82.0                  |
| *Hymenolepididae gen. sp.*       | Cyclophyllidea/Hymenolepididae | *Cygnus cygnus* (China)         | KU980902.1             | 35.5           | 83.0                  |
could not be solved using nuclear loci (Okamoto et al., 1995; Zhang & Hewitt, 1996; Awwad et al., 2001; Macnish et al., 2002). In this study, mtCOXI was used to identify the recovered cestode parasite phylogenetically, in agreement with the findings of Lecanidou et al. (1994) who used mtCOX subunits genes (I and II) to determine the phylogenetic relationships between related organisms with rapid evolutionary rates. Similar to Guo’s (2016) method, this study used the maximum likelihood method to build the phylogenetic tree along with representatives of Pseudophyllidea (Diphyllobothriidae) and Cyclophyllidea.

Fig. 5. Sequence alignment of the mtCOX1 gene of H. nana with the most closely related hymenolepidid species. (Only variable sites are shown. Dots represent bases identical to those of the first sequences, and dashes indicate gaps).
(Anoplocephalidae, Hymenolepididae, and Taeniidae) with strongly supported independent clades. Hymenolepididae exhibited a sister-group relationship with Anoplocephalidae, consistent with the findings of studies based on 18S rDNA (Mariaux, 1998; Von Nickisch-Rosenegk et al., 1999), 12S rDNA (Von Nickisch-Rosenegk et al., 1999), and COX genes (Haukisalmi et al., 1998). This was also consistent with the cladistic analysis results of Hoberg et al. (1999) based on morphological characters. In addition, our data support the findings of Al Quraishy et al. (2019), which showed the closely related arrangement of Mesocestoides in Cyclophyllidea with Taeniidae, Hymenolepididae, and Anoplocephalidae.

Our results confirm the paraphyletic origin of the genus *Hymenolepis*, consistent with the findings of Haukisalmi et al. (2010) and Kandil et al. (2010), indicating that both Taeniidae and Hymenolepididae are paraphyletic. Our results, however, contradict Macnish et al. (2002), who reported the *Hymenolepis* genus’ monophyly. In our analysis and comparison of mtCOXI with the known sequences of other cyclophyllidean cestodes, the sequences of the query showed close similarity to that of the present and previously described *H. nana* (gb| AP017666.1), with a high bootstrap value of 96%. These results are consistent with the hypothesis of Hillis & Bull (1993), Campbell & Beveridge (1994), and Palm (2004) who found the general rule in the phylogenetic analysis; where the bootstrap value for a given interior branch of a phylogenetic tree is 70% or higher, the topology for that branch is considered reliable or correct. In this study, *H. nana* was found to be more closely related to *H. diminuta* than *H. microstoma*; this contradicted the data obtained by Nkouawa et al. (2016), who built phylogeny using 28S rDNA genomic regions and mtCOX1 marker regions, and demonstrated the genetic relationship between *H. nana* and *H. microstoma*. In contrast, the appearance of both *H. diminuta* and *Pseudacteon crawfordi* in the same clade substantiates the phylogenetic relationship between the two taxa, as clarified by Cheng et al. (2016). The current phylogenetic analyses revealed that the relationship between *Hymenolepis* species and *Rodentolepis fraterna* (gb| IN258053.1) and *Hymenolepididae* gen. sp. (gb| KU980902.1) was unexpected, as they represent different rostellar forms. As described by Haukisalmi et al. (2010), this is due to the loss of rostellar hooks and functional rostellum by *Hymenolepis* species, after colonization from arvicoline rodents. This study further supports the taxonomic position of the recovered hymenolepidid species, with a unique genetic sequence deeply embedded in a genus including the species described of *H. nana*, as a putative sister taxon.

**Conclusion**

Our recent field study provides new insights into the rapid detection of rodent-infecting hymenolepidids and systematic and phylogenetic analyses. The mitochondrial gene of *H. nana* has also been found as a unique sequence to confirm its taxonomic position in the Hymenolepididae family. In addition, our findings

Fig. 6. Molecular phylogenetic analysis carried out using the Tamura–Nei model based on the maximum likelihood method. The percentage of trees shown above the branches that clustered together the related taxa. Initial tree(s) for heuristic search was automatically obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the maximum composite likelihood (MCL) approach, then selecting the topology with the higher log-likelihood value. The tree is drawn to the scale, comparing branch lengths as the number of substitutions per site.
indicated that laboratory mice could be considered as possible natural reservoirs of different cestode parasites. Further investigation should focus on analyzing different genes that can assist in clarifying the phylogenetic relationships between Hymenolepididae.

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Conflict of interest. The authors declare that they have no conflict of interest.

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