Morphological and Molecular Characterization of *Aspiculuris tetraptera* (Nematoda: Heteroxynematidae) from *Mus musculus* (Rodentia: Muridae) in Saudi Arabia

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Abstract: *Aspiculuris tetraptera* a pinworm of mice, is an important parasite in institutions with mice colonies for both research and teaching purposes. Infection with this parasite has impact on biomedical research. This is likely due to the availability of the parasite’s eggs in the environment, therefore can easily be transmitted and infection is generally asymptomatic. No information regarding the prevalence, morphology or phylogeny is available on *A. tetraptera* from Saudi Arabia. A group of 50 laboratory mice were investigated for the presence of *A. tetraptera*. Worms were described morphologically and molecular characterization was attempted using 18S rRNA and Cytochrome Oxidase Subunit I genes. The prevalence of *A. tetraptera* infestation in the laboratory mice examined was found to be 46%. Morphological description indicated that the worms belong to *A. tetraptera* and this was confirmed by molecular characterization. Both regions studied has shown that the worm under investigation grouped with *A. tetraptera*. 18S rDNA sequences obtained in the present study showed high identity with sequences from *A. tetraptera* while *COI* sequences showed intraspecific variation resulted into two haplotypes from the isolates in the present study. *A. tetraptera* was recorded for the first time from Saudi Arabia. Molecular characterization has shown, based on the *COI* sequences, that the Saudi isolates of *A. tetraptera* are distinct.

**Keywords:** *Aspiculuris tetraptera*, 18S rRNA, Cytochrome Oxidase Subunit I, *Mus musculus*, Saudi Arabia.
Introduction

Members of the genus *Aspiculuris* belong to the Oxyurid nematodes are inhabitants of the colon and caecum of rodents and they possess 3 pairs of alae in the tail region of male worms.

To date, 20 species belong to the genus *Aspiculuris* have been recognized including the recently described *A. huascaensis* and *A. tianjinensis* [1-3]. This group of worms are morphologically designated as two main groups based on the appearance of the cervical alae [4]. The first group is characterized by having cervical alae interrupted with the posterior ends and forming acute angle towards the anterior. This group composed of 14 species including *A. tetraptera*.

Members of the second group, are characterized by having the cervical alae gradually decrease in width, joining the wall-body or to the lateral alae if it is present and this groups included 6 species [3,5-7]. *A. tetraptera* (Nitzsch, 1821) is a natural and common intestinal parasite of the *Mus musculus* which occur throughout the world. The worm was detected in other rodents’ species such as the European bank voles (*Myodes glareolus*) with high prevalence and heavy worm infestation [8,9]. It has also been recorded as an incidental parasite from the wood mouse (*Apodemus sylvaticus*) due to the low numbers recovered [10-12]. Observations on naturally acquired oxyurid infections and experimental infections with *A. tetraptera* clearly show the high variability of resistance/susceptibility between different mouse strains from the wild [13-16].

The life cycle of *A. tetraptera* is a direct one and infection occurs after ingestion of eggs by the host with a prepatent period of 21-35 days [17]. The first stage larvae stay for a week in the submucosa of the colon and develop to the third larval stages which return to the lumen of the colon to develop to adulthood [17,18]. Therefore, infection in the laboratory mice cannot be
prevented and infected animals will remain infected unless treated. Although infected animals  
may not show clinical signs in immunocompetent laboratory mice, various studies have shown  
effects of some parasites including *A. tetraptera* associated with the immune response,  
reduction of hemoglobin, RBCs count and serum albumin which may adversely affect the  
findings of the experiments [19-22].

Helminths parasites of rodents reported from Saudi Arabia included nematodes as *Hymenolepis  
nana, Mastophorus muris, Trichuris muris* and *Dentostomella tamimi* from the spiny mouse  
(*Acomys dimidiatus*); *Trichsomoides crassicauda* from the Wistar rat (*Rattus rattus*); *Syphacia  
spp. Trichuris* spp., *Hymenolepis diminuta*, as well as intermediate stages *Cysticercus fasciolaris*  
of the cat and other carnivores’ tapeworm *Hydatigera taeniaeformis* from *R. rattus* and *R.  
norvegicus* [23-27].

Research on *A. tetraptera* in Saudi Arabia is lacking and no previous studies have been  
conducted in this worm which infect laboratory mice. No morphological or genetic information  
regarding *A. tetraptera* from Saudi Arabia despite the extensive use of laboratory mice in  
various experiments. Only few DNA sequences from *A. tetraptera* are available in the GenBank.  
Hence, the current study was undertaken to describe *A. tetraptera* from the laboratory mice  
and to characterize the recovered worms using both 18S rRNA gene as a nuclear gene and  
cytochrome oxidase subunit I as a mitochondrial gene. The phylogenetic relationship between  
the worms isolated from Saudi Arabia were investigated with those worms which were  
reported from other parts of the world.

**Materials and Methods**

**Specimens and morphological investigations**
The study protocol was following guides on the care and use of laboratory animals approved by the Ethical Committees of the King Saud University. This work was conducted at the Department of Zoology, College of Science, King Saud University. A total of 50 laboratory adult mice weighing 25-30 g (27 ± 1.1 mean ± SD) intended for students’ routine work were examined for parasites. This group of animals is from the same source where experimental animals for research purposes are being obtained. Laboratory mice were euthanized using an overdose of CO₂ (flow rate 3 L/min) and the CO₂ was continued for 1 min after the breathing stops. The animals were dissected by students for educational purposes and the intestinal tract was removed and examined for worms. Nematodes were recovered from the intestinal contents of animals after being dissected for Biology classes. Adult nematode worms were collected, placed in 70% ethanol, and were left in the refrigerator (4 °C) overnight. The worms were then preserved in 5% glycerin alcohol. The worms were cleared in lactophenol, examined microscopically. Morphological characteristics of the worms as well as the measurements were done using an ocular micrometer. 10 male and 10 female worms were used in the investigation. All measurements are in micrometer (µm). Identification of recovered worms was achieved following the keys given in Yamaguti together with Anderson and others [28,29].

DNA extraction from nematodes and Polymerase Chain Reaction

Worms were washed in distilled water several times to remove the alcohol and were taken carefully into plastic Eppendorf tube. DNA from individual worms which were identified as A. tetraptera was extracted using the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany).
Germany) following the manufacturer’s instructions. Extracted DNA was eluted in 100 μl of the elution buffer.

The polymerase chain reaction amplifications were conducted using a Multigene™ thermocycler (Labnet International, Inc., Edison, NJ, USA). The primers used for amplification of the whole of 18S RNA gene were AP1 (5’-AACCTGGTTGATCCTGCCAGT-3’) as a forward primer and Ap2-R (5’-TGATCCTTCTGAGGTTCATCCCTAC -3’) as a reverse primer and the primer GW1-F (5’-TTTGGTAGNCTGA -3’) was used together with AP2 to amplify a fragment of 1000 base pair (bp) at the second half of the 18S rRNA gene [30]. Another set of primers was also used to amplify partial 18S rDNA region which included Nem 18SF (5’-CGCGAATRGCCTATTACAACAGC-3’) as a forward primer and Nem 18SR (5’-GGGGGATGTCTGATCGC-3’) as a reverse primer as indicated by Floyd and others [31]. The forward primer binds at a site around 100 bp inward from the 5’ end of the gene, and the reverse primer at around 700 bp inward from the 3’ end. The mitochondrial Cytochrome C Oxidase subunit I gene (COI) was amplified using the universal primers LCO1490 (5’-GGTCAACAAATCATAAAGATATTGG-3’) as a forward primer and HC02198 (5’-TAAACTTCAGGGTGACCAAAAAATTCA-3’) as a reverse primer [32]. PCRs were conducted in a reaction mixture of 25 μl containing 5 μl of the worm DNA, 5 μl of the 5x reaction buffer, 0.1 μm of each primer (forward and reverse for each region), 1 unit/μl of Taq polymerase (Bioline, London, UK), and total volume was made to 25 μl by adding PCR grade water. A negative control containing PCR grade water was included in each PCR. The PCR amplification conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 40 cycles, denaturation at 94°C for 30 s, annealing for 30 s on 55°C for 18S (using AP1, AP2 and
GW1 and AP2 primers) and 50 °C for COI, 54 °C for 18S (using Nem18SF and Nem 18SR primers) followed by 30 s extension at 72°C and a final extension at 72°C for 5 min.

**DNA detection, sequencing and phylogenetic analysis**

PCR products were confirmed using gel documentation system Bio Pyramid (MeCan Imaging Inc., Saitama, Japan) when subjecting the agarose gel stained with ethidium bromide to ultraviolet light in the transilluminator. Digital images of the PCR products were obtained and PCR products were sequenced using Macrogen sequencing facility (Macrogen Inc, Seoul, South Korea).

Phylogenetic relationships between the sequences from A. tetraptera and related nematodes species were inferred using 18S rDNA, and COI loci available in GenBank database. The analysis was performed using Bayesian Inference (BI) using Mr Bayes [33] and Maximum Likelihood (ML) available in MEGA 7.0 software using the trematode Clonorchis sinensis as an outgroup [34]. Markov chain Monte Carlo (MCMC) chains were run for 2,000,000 generations, log-likelihood scores were plotted, and the final 75% of trees were used to produce the consensus trees. The relevant COI sequences available in the GenBank of A. tetraptera were obtained and haplotype network was conducted using Population Analysis with reticulate Trees (PopART) software available at [http://popart.otago.ac.nz](http://popart.otago.ac.nz) using Templeton, Crandall and Sing (TCS) option [35]. COI sequences were translated into amino acids using MEGA 7.0 software to check for possible amplification of pseudogenes. Sequences from the GenBank for both 18S rDNA and COI regions which are used in the present investigation are presented in Table 1.

**Results**
The male and female nematode worms collected in this study were described and diagnosed on the basis of morphological and morphometric characteristics and preliminarily identified as *Aspiculuris tetraptera*. Worms show that cervical alae abruptly interrupted, forming an acute angle, ending at the oesophageal bulb (Fig. 1). Measurements were taken, compared with some previous studies and presented in Table 2. The adult worms of *A. tetraptera* were found in the large intestines of 23 (46%) out of 50 individuals examined.

Polymerase chain reaction products were obtained from some specimens, which were identified as *A. tetraptera*, and the expected sizes for each primer pair which amplify 18S rDNA (4 specimens) and partial COI (7 specimens) regions were detected on agarose gel electrophoresis. 18S rDNA sequences were obtained from using the primers AP1 and AP2 as well as GW1 and AP2 while attempts to amplify 18S rDNA region using primers Nem 18SF and Nem 18SR were unsuccessful. Sequence analysis of the sequences obtained from both 18S rDNA and COI regions confirmed the identity of worms under investigation as *A. tetraptera*. The sequences (4 sequences) obtained for the nuclear 18S rDNA region using the primers AP1, GW1 and AP2 were identical and they are 1680 bp in length and a representative sequence was deposited in GenBank database under the accession number MT755640. The length of sequences (7 sequences) from the mitochondrial COI region was 629 bp and deposited in GenBank database under the accession numbers MT621040-MT621046.

Bayesian Inference and Maximum Likelihood phylogenetic trees showed that the representative 18S rDNA sequence of *A. tetraptera* resulted from the present study formed a distinct clade containing Heteroxynematidae of Oxyuroidea with *A. tetraptera* forming a distinct subclade with a strong bootstrap value support (Fig. 2).
Sequences obtained from the COI region were 629 bp. However, there was intraspecific variation in the sequences obtained from the COI region in the present study and the identities of sequences ranged between 99.8% to 100%. The variation within the sequences obtained in the present study and those from the GenBank ranged from 97.5% to 98.6%. The A/T content of the sequences obtained in the present study ranged between 64.87% to 65.03%. There were changes in 26 sites of the alignment, 15 of them were transversions while 11 were transitions (Table 3). The alignment composed of sequences from A. tetraptera from different regions; KT764937 (China), KP338608 (India), LC038093 (Japan), KF444292, 444293, 444298, 444305, 4444306 (China). The COI sequences obtained in the present clustered with those of A. tetraptera. However, the group separated in to two subclades on containing the sequences obtained during the present study and other containing sequences from the GenBank.

Phylogenetic tress using BI and ML showed that sequences of A. tetraptera in the present study are related to sequences from A. tetraptera available in the GenBank, forming a distinct group (Fig. 3).

A haplotype network of COI gene diversity in A. tetraptera isolates is shown in Fig. 4. The number of sites of the COI region included 629 bp and sites with alignment gaps (234 bp) were not considered, hence the analysis was performed on 395 bp. Total number of mutations at the sites analysed was 16 suites (Fig. 4). Considering the whole region an additional haplotype from the Saudi isolates (i.e. MT621044) of A. tetraptera may results as there was some variation in the sequences which has not been considered when eliminating the gaps as presented in Table 3.

Discussion
In many animal facilities, mice and other rodents’ colonies are either infected with helminthic parasites or become infected in the laboratories where experiments take place. Therefore, examining laboratory mice intended for laboratory experiments is important. This report constitutes the first detection of *A. tetraptera* from laboratory mice in Saudi Arabia. Morphological as well as morphometric characteristics showed that the species recovered from mice autopsied at the Department of Zoology, College of Science, King Saud University, resembles *A. tetraptera* as has been described previously by [6,36]. There were slight morphometric differences between other *A. tetraptera* described from other studies [37]. Morphometric variation in the worms *A. tetraptera* has been noticed from studies from the same region. On separate studies some investigators recorded variation in lengths of male and female *A. tetraptera* [37-39]. The lengths of the worms reported in the present study, however, overlapped with these studies.

The prevalence of *A. tetraptera* infection in the laboratory mice, which are used in research, was found to be 46% which is considerable high prevalence. Previous reports on the prevalence of *A. tetraptera* in laboratory mice had shown considerably low prevalence to what we have detected in the present study. Bazzaro and others [40] detected a prevalence of 8.5% and Kataranovski and others [41] detected a prevalence of 12.8%. On a recent study from Egypt Abdel-Gaber and others [37] detected as high as 56% of *A. tetraptera* prevalence of *A. tetraptera* infection in the mice investigated. It has been reported that infections with the rodents’ oxyurid nematodes; *A. tetraptera* and *S. obvelata* generally influenced by the age of the host and infection with the first worm affect older animals while the later affects younger animals occur in young mice [42,43]. Reporting of
high prevalence in our study and Abdel-Gaber and others [37] study may be explained by the fact that adult mice were investigated in both studies as has been reported before [42,43].

Molecular characterization of the helminths’ parasites investigated in the present study have clearly shown from the 18S rDNA sequences obtained that these helminths possess sequences which are related to three sequences belonging to *A. tetraperta* which are available in GenBank. These sequences were from South Africa (2 sequences) whereas one sequence from China [44]. The sequences obtained from the 18S rDNA region showed high identity to the sequences obtained from the nematode *A. tetraperta* which are available in GenBank. Only three sequences from the 18S rDNA region studied of *A. tetraperta* are available in GenBank. These sequences have the following accession numbers: KY462827, KY462828 (from South Africa) and MH215350 (From China). At position 1 of the alignment sequence MH215350 showed an A while the other sequences including the sequences from the present study showed an G. At position 56 sequences from the present study showed an A unlike a G on other sequences. Also sequences from the present study showed an A as opposed to a T in other sequences at position 162. At position 1652 sequences of the present study showed a T instead of an A in sequence KY462827. The three sequences in GenBank were 98.0 to 99.7% identical to the sequences reported in the present study.

Our phylogenetic analysis of the 18S rDNA sequences supported grouping the superfamily Oxyuroidea in a single clade which included 18S rDNA sequences of *A. tetraperta* and that of *Ozolaimus linstowi*. It agreed with previous taxonomic grouping of related nematodes [45,46]. A recent study by Abdel-Gaber and others [37] who studied the morphology of *A. tetraperta* and claimed that sequences of the 18S rDNA was studied has shown that they have dealt with
internal transcribed spacer regions rather than 18S rRNA gene. Furthermore, they have used the primers indicated by Floyd and others [31] which amplify partial 18S rRNA gene and which has not resulted into products in the present study despite changing the PCR conditions. It is unclear if they have used different primers to amplify the ITS1 and the ITS2 regions in their study or the same primers amplified the region which they reported. Floyd and others [31] designed two forward and three reverse primers which they found suitable for amplifying only nematodes DNA and not fungal DNA. However, they have released only one primer pair which they have used in their study and claimed that these primers are suitable for amplifying nematodes. It is likely that DNA of A. tetraperta is not good candidate to be amplified using these primers. Phylogenetic analysis has clearly grouped the 18S rDNA sequence reported in the present study with those sequences from A. tetraperta in one distinct clade including Heteroxynematidae within Oxyuroidea.

The COI sequences obtained in the present study have shown some intraspecific variation with the similar sequences generated from A. tetraperta from other regions. The A/T content of the sequences obtained ranged between 54.55% to 65.03% which was consistent with that of mitochondrial genes from some other helminths’ parasites [47,48].

Sequences obtained in the present study together with related sequences have shown that there were 6 haplotypes (I to VI) based on sequence variation on the COI sequences with haplotype diversity of Hd: 0.5379. In the GenBank there were 30 COI sequences which were compared to assign different haplotypes. Haplotype I was detected in one sequence obtained from India (KP338608), haplotype II was in the sequence (KT764937) from China, Haplotype III was in 5 sequences (MT621040, MT621041, MT621042, MT621043 and MT621044) from the
present study, haplotype IV was from 2 sequence (MT621045 and MT621046) from the present study, whereas haplotype V included 20 individuals (LC038093; from Japan and KF444293 to KF444311 from China) and haplotype VI was from a single individual (KF444292) from China.

Isolates from Saudi Arabia have shown two different haplotypes. Other haplotypes were from isolates from China, Japan and India. It appears that isolates from Saudi Arabia are distinct, however, there was no significant morphological differences which may suggest the worms could be other than *A. tetraptera*. Therefore, further investigation is required to fully resolve the relationship between the morphological features and the evolutionary history of *Aspiculuris* species from the two main known morphological groups. Behnke and others [36] studied the phylogeny of different species of *Aspiculuris* from different hosts. They have reported *A. tianjinensis*, which has previously been reported from China, from the bank vole (*Myodes garleolus*) and *A. tetraptera* from *Mus musculus*. Their investigation revealed that there is distinct differentiation between different worm species recovered from different hosts suggesting that *Aspiculuris* spp. can be regarded as a marker for host evolution. The *COI* sequences studied were from 25 isolates from *Aspiculuris* spp. (11) were from *A. tetraptera*, (8) were from *A. tianjinensis*, (4) were from *A. dinniki*, (1) were from each of *A. Africana* and *A. americana* [36]. The region they studied was only 143 pb and unfortunately it was not included in the sequences obtained in the present study, therefore, it was not possible to compare our results with what they have found. In the short fragment they studied they found 18 SNPs which could differentiate between different species studied. The sequences variation in the *A. tetraptera* was not high and only two haplotypes were found. There was no sequence variation in all isolates of *A. tianjinensis* and they were identical. Interestingly the 4 isolates of *A. dinniki*
showed high variation and resulted in 4 haplotypes. In our study we found 26 SNPs with in A. tetraperta sequences which indicates that the COI region may be a better marker studying the population genetics of different Aspiculuris species. Three haplotypes from the COI sequences were reported from the Saudi strains which were different from those isolated from China, India and Japan.

In Behnke and others [36] study only three of the 18 SNPs resulted in changes to the inferred amino acid sequence, whereas in the present study 16 of the SNPs have resulted in amino acid sequence change.

In conclusion, A. tetraperta is a common intestinal nematode in laboratory mice. Since it has considerably high prevalence in the present study, there a need for a strict control and prevention measures in these colonies. The haplotypes of A. tetraperta in the present study were distinct from other haplotypes reported from other parts of the world.

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Authors’ contribution

SAO and OBM conceived the study; SAO, JMA, MA and AHA obtained the samples and conducted laboratory work; SAO and OBM performed the analysis of data; OBM drafted the manuscript; SAO, JMA, MA and AHA read, commented and approved the manuscript.
Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Data availability statement

Data associated with this paper are available by contacting the corresponding author.

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Legends of Figures:

Figure 1. Features of Aspiculurus tetraptera. A photomicrograph showing a male Aspiculuris tetraptera whole worm cleared using lacotphenol (A). The anterior part of male A. tetraptera (B) showing diagnostic features; the cephalic vesicle (CV) or inflation with lips and papillae, the muscular oesophagus (MOe) and the bulbar region of the oesophagus (OeB) together with the characteristic cervical ala (CA).

Figure 2. Molecular phylogenetic tree using 18S rDNA data and generated by Bayesian inference (BI) and maximum-likelihood (ML) methods, showing phylogenetic position of A. tetraptera reported from Saudi Arabia. Utilizing Clonorchis sinensis as an outgroup using sequences of related taxa from the GenBank. Bootstrap values indicated in the branches included the posterior probability (BI/ML). Values shown are those > 50%. Representative sequence from A. tetraptera from Saudi Arabia in indicated in bold.

Figure 3. Molecular phylogenetic tree using cytochrome c oxidase subunit I (COI) data and generated by Bayesian inference (BI) and maximum-likelihood (ML) methods, showing phylogenetic position of isolates of A. tetraptera reported from Saudi Arabia. Utilizing Clonorchis sinensis as an outgroup using sequences of related taxa from the GenBank. Bootstrap values indicated in the branches included the posterior probability (BI/ML). Values shown are those > 50%. Sequences from A. tetraptera from Saudi Arabia in indicated in bold.
Figure 4. Network analysis of partial mitochondrial cytochrome c oxidase subunit I (COI)

haplotypes of A. tetraptera detected in the present study and those available in the GenBank.
The analysis was performed using Population Analysis with Reticulate Trees (PopART)
software using TCS option for haplotypes presentation. Marked haplotypes are those
detected in the current study.
Table 1. DNA sequences from GenBank and their accession numbers of organisms used in the analysis. Sequences obtained from the present study are in bold.

| Organism                        | Accession Number | Organism                        | Accession number |
|--------------------------------|------------------|--------------------------------|------------------|
| *Aspiculuris tetraptera*        | MT755640         | *Aspiculuris tetraptera*        | MT621040         |
| *Aspiculuris tetraptera*        | KY462827         | *Aspiculuris tetraptera*        | MT621041         |
| *Aspiculuris tetraptera*        | KY462828         | *Aspiculuris tetraptera*        | MT621042         |
| *Blattophila peregrinata*       | MH215350         | *Aspiculuris tetraptera*        | MT621043         |
| *Clonorchis sinensis*           | MK450527         | *Aspiculuris tetraptera*        | MT621044         |
| *Contracaecum eudyptulae*       | EF180072         | *Aspiculuris tetraptera*        | MT621045         |
| *Contracaecum microcephalum*    | AY702702         | *Aspiculuris tetraptera*        | KP338608         |
| *Dirofilaria immitis*           | AB973231         | *Aspiculuris tetraptera*        | KT764937         |
| *Dirofilaria repens*            | AB973229         | *Clonorchis sinensis*           | YP002640631      |
| *Hammerschmidtia keenyi*        | KX752429         | *Dirofilaria immitis*           | NP954717         |
| *Onchocerca cervicalis*         | DQ094174         | *Oxyuris equi*                 | YP009142700      |
| *Ozolaimus linstowi*            | KJ632671         | *Passalurus ambiguus*           | KT879302         |
| *Spirocerca lupi*               | AY751497         | *Setaria digitata*             | AQM38780         |
| *Streptopharagus sp.*           | HM067977         | *Syphacia obvelata*            | MH427232         |
| *Toxascaris leonina*            | JN256984         | *Syphacia obvelata*            | MH427234         |
| *Toxocara canis*                | JN256976         | *Syphacia obvelata*            | MH427235         |
| *Toxocara cati*                 | JN256973         | *Trypanoxyuris minutus*         | MF379241         |
|                                 |                  | *Trypanoxyuris minutus*         | MF379248         |
|                                 |                  | *Wellcomia siamensis*           | YP004927932      |
Table 2. Morphological features and measurements of *A. tetraptera* recorded in the present study compared with previously described results. Only lengths of worms are given in millimetres (mm) while other measurements are in microns (μm).

| Morphological feature       | *A. tetraptera* (This study) | *A. tetraptera* [36] | *A. tetraptera* [6] |
|----------------------------|-----------------------------|----------------------|---------------------|
|                            | Male | Female | Male | Female | Male | Female |
| Length (mm)                | 2.3 - 2.7 | 2.6 - 3.4 | 1.6 - 3.7 | 3.4 - 4.5 | 2.7 | 3.8 |
| Width                      | 150-194 | 190-230 | 136-238 | 167.5-272 | 110 | 200 |
| Cephalic vesicle length    | 66.4-85.5 | 77.5-90.3 | 67-99 | 92.4-113.9 | 80 | - |
| Cephalic vesicle width     | 88.0-92.0 | 90.5-99.2 | 68-102.3 | 93.6-113.9 | 90 | - |
| Oesophagus length          | 315.2-350.3 | 330-380 | 301-408.7 | 395.3-502.5 | 380 | 430 |
| Oesophageal bulb length    | 120.5-135.4 | 128.6-143.4 | 105.5-148.5 | 132-184.4 | 140 | 160 |
| Oesophageal bulb width     | 86-102 | 95-112 | 66-89.1 | 82.5-115.5 | 90 | 130 |
| cervical alae length       | 300-320 | 325-350 | 244-368.5 | 321.6-402 | 260 | 350 |
| Nerve ring                 | - | - | 132-148.5 | 99-145.2 | 100 | 130 |
| Tail                       | 127-152 | 184-250 | 167.5-207.7 | 402-643.2 | 170 | 550 |
| Vulva to anterior          | - | 1100-1400 | - | 1273-1700 | - | - |
| Egg                        | - | 80-97.5 x 35x50 | - | 82.5-92.4 x 36.3-49.5 | - | - |
Table 3. Variable site of COI sequences obtained from *A. tetraptera* in the present study (7 sequences, **in bold**) compared with sequences obtained from the GenBank (5 sequences). (-) indicated that sequences were not covering the desired position.

| SITE | Taxon   | 100 | 113 | 115 | 116 | 132 | 134 | 170 | 217 | 247 | 295 | 302 | 335 | 352 | 365 | 382 | 493 | 514 | 529 | 646 | 652 | 667 | 675 | 713 | 716 | 725 | 727 |
|------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|      |         |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| KP338608 | A       | A   | T   | T   | A   | C   | T   | T   | A   | C   | A   | A   | G   | T   | G   | A   | A   | G   | A   | A   | C   | A   | G   | T   | T   | C   |
| KT764937 | C       | G   | T   | T   | G   | G   | G   | T   | T   | G   | T   | A   | T   | A   | T   | T   | T   | C   | C   | T   | T   | G   | T   | T   | C   |
| MT621040 | C       | G   | T   | T   | T   | G   | T   | T   | A   | G   | A   | A   | G   | T   | G   | A   | A   | A   | G   | A   | A   | A   | G   | A   | T   | C   | T   |
| MT621041 | C       | G   | T   | T   | T   | G   | T   | T   | A   | G   | A   | A   | G   | T   | G   | A   | A   | A   | G   | A   | A   | A   | G   | A   | T   | C   | T   |
| MT621042 | C       | G   | T   | T   | T   | G   | T   | T   | A   | G   | A   | A   | G   | T   | G   | A   | A   | A   | A   | G   | A   | A   | A   | A   | G   | A   | T   | C   |
| MT621043 | C       | G   | C   | C   | T   | G   | T   | C   | A   | G   | A   | A   | G   | T   | G   | A   | A   | G   | A   | A   | A   | G   | A   | A   | T   | C   | T   |
| MT621044 | C       | G   | T   | T   | T   | G   | T   | T   | A   | G   | A   | A   | G   | T   | G   | A   | A   | A   | G   | A   | A   | A   | G   | A   | T   | C   | T   |
| MT621045 | C       | G   | T   | T   | T   | G   | T   | T   | A   | G   | A   | A   | G   | T   | G   | A   | A   | A   | G   | A   | A   | A   | G   | A   | A   | C   | C   |
| MT621046 | C       | G   | T   | T   | T   | G   | T   | T   | A   | G   | A   | A   | G   | T   | G   | A   | A   | A   | G   | A   | A   | A   | G   | A   | A   | C   | C   |
| LC038093 | -       | -   | -   | -   | -   | -   | -   | -   | -   | -   | A   | G   | T   | G   | A   | A   | G   | A   | A   | A   | G   | A   | A   | G   | T   | C   |
| KF444293 | -       | -   | -   | -   | -   | -   | -   | -   | -   | -   | A   | G   | T   | G   | A   | A   | G   | A   | A   | A   | G   | G   | T   | T   | C   |
| KF444292 | -       | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | T   | G   | T   | G   | A   | A   | G   | A   | A   | A   | G   | G   | T   | T   | C   |
