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RESEARCH ARTICLE

Molecular characterisation and genetic affinities of Cyclophyllidean cestodes infecting wild rats in Peninsular Malaysia

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ABSTRACT

Cestode infections is widely reported in rodents, however species identification remains problematic due to the genetic or interspecies variation. Therefore, this study was aimed to verify the Cyclophyllidean parasites recovered from wild rats captured from different forest types using molecular based methods. Maximum-likelihood (ML) and neighbour-joining (NJ) trees were constructed inferred from 18 small subunit nuclear ribosomal RNA gene (18SrDNA) and mitochondrial cytochrome c oxidase subunit one gene (COX1) sequences of cestode worms recovered from 124 individuals from four rat species. Sequences obtained from both *Hymenolepis diminuta* and *Hydatigera parva* represents the first records in Malaysia. All the sequences were successfully amplified with product with total length of 205 and 1202 base pairs (bp), respectively. Three cestode species from the Family Hymenolepididae (*Hymenolepis diminuta*) and Family Taeniidae *(Hydatigera parva*; *Hydatigera taeniaeformis*) were successfully characterized using phylogenetic analyses and haplotype networking. Phylogenetic analysis showed that *H. diminuta*, *Hydatigera parva* (*Hy. parva*) and *Hydatigera taeniaeformis* (*Hy. taeniaeformis*) formed its own monophyletic clade in 18SrDNA analyses. Results also showed that *Hy. taeniaeformis* shared the same haplotype group with *Hy. taeniaeformis* from China (COX1) and linked with *Hy. taeniaeformis* from Japan (18SrDNA) while the Malaysian *H. diminuta* clearly formed a separate haplotype and networked with other regions. The Malaysian *Hy. parva* isolation, on the other hand, appeared to be genetically distinct from the European *Hy. parva* (Spain) strain, but closely linked to the local isolates. Molecular methods employed successfully improved in the detection of complex species in this group. The findings showed that molecular data can be useful to deeply study intra-specific variation in other cestode worms.

Keywords: Zoonoses; Taeniidae; Hymenolepididae; genetic divergence; murids.

INTRODUCTION

Cestoda is a group of obligate endoparasites that exhibit a variety of body forms, life cycles and host relationships. The largest taxonomic community within the Cestoda is the order Cyclophyllidea, comprising of 16 recognized families such as Taeniidae Ludwig, 1886 and Hymenolepididae Ariola, 1899 (Mariaux *et al*., 2017)

With a total of 850 species (620 in birds and 230 in mammals), the family Hymenolepididae is the richest in the number of species of all the cestode families (Czaplinski & Vaucher, 1899). *Hymenolepis diminuta* (rat tapeworm) and *Hymenolepis nana* (dwarf tapeworm) are a neglected zoonotic disease in humans transmitted by rats. In addition, there are four genera in the family Taeniidae, namely *Echinococcus* Rudolphi (1801); *Hydatigera* Lamarck (1816); *Taenia* Linnaeus (1758); and *Versteria* (Nakao *et al*., 2013). *Taenia* species causes intestinal diseases known as taeniasis and metacestode larval to cause cysticercosis and coenurosis in domesticated animals and humans.

Primarily, most of these studies utilized conventional methods for identification such as optical microscopy or morphological criteria. Difficulties in taxonomic identification associated with conventional methods have been highlighted such as morphological similar species and diminutive parasite size making it difficult to stain and recognize. However, the advancement in molecular methods has enabled us to overcome these issues and seen as the future for the identification of poorly known parasite groups, including cestodes (Lavikainen *et al*., 2008; Galimberti *et al*., 2012; Yan *et al*., 2013; Poon *et al*., 2017). In addition, phylogenetic studies on cestodes infecting rats have not been well established and to date, information on local cestodes is still lacking in the GenBank.

Therefore, in this study, universal primers of 18 small subunit nuclear ribosomal RNA gene (18SrDNA) and mitochondrial cytochrome c oxidase subunit one gene (COX1) were utilized as a genetic marker to identify and characterize the cyclophyllidean worms recovered from the wild rat population collected at several locations in Peninsular Malaysia. Several cestodes species in the

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GenBank were referenced to verify the genetic relationships and determine haplotype networking.

MATERIALS AND METHODS

Ethical statement

All protocols in this study involving handling of animals strictly follow in the Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011). This study was approved by the Institutional Animal Care and Use Committee of the Universiti Malaya (UM IACUC) with ethics clearance number: S/18092020/28072020-01/R. Research permits were issued by the Department of Wildlife and National Park (DWNP) of Peninsular Malaysia and Department of Forestry Malaysia and with reference number JPHL&TN(IP): 100-34/1.24 Jld 14(66) and JH/100 Jld. 23(3), respectively.

Study areas and samples collection

This study was carried out in 3 forest types: forest reserve, recreational forest, and modified forest (agriculture land) as shown in Figure 1 and Table 1. A total of 100 wire cages sized 28 cm × 15 cm × 12 cm were laid out for 5 days of trapping at various elevations (i.e., not more than 300 meter above sea level) in different microhabitats as along the vegetation, forest trails and under the trunk, stream, or river.

Helminth collection

Captured rats were immediately euthanized and *postmortem* examination was conducted according to the protocol described by Herbreteau *et al*. (2011). Necropsy was conducted with the removal of the organs such as liver and gastrointestinal tract including oesophagus, stomach, small intestine, large intestine, and ceacum and preserved individually in 70% alcohol for further helminthological observation. Majority of adult worms were recovered from the small intestine while cysticercus were generally found on the liver. However, in one rat, some cysticerci were recovered underneath cheek muscles. All procedures followed according to protocol provided by Henttonen & Haukisalmi (2008). All tapeworms obtained were preserved in a vial with 70% ethanol and stored at -20°C immediately prior to DNA extraction.

DNA Extraction and amplification

Genomic DNA was extracted from worm tissue (a whole individual for small specimens or 1-2 proglottids for the larger specimens) using the Vivantis GF-1 extraction kits. DNA extraction followed the manufacturer's protocols (Vivantis Technologies Sdn. Bhd). The extracted DNA was then stored in -20°C until further use. The NanoDrop™ 2 000 Spectrophotometer (Thermo Scientific, USA) was used to determine the purification of the extracted genomic DNA with A260/A280 nm ration using 1:10 (DNA: buffer) dilution (Ikbal *et al*., 2019). Two primer sets; 18SrDNA and COX1 genes were used (see Table 2).

A total 50 µl reaction mixture containing 2 µl of the DNA template, 2 µl of each primer, 19 µl of ultra-pure water, 25 µl of 2× Power Taq PCR MasterMix (BioTeke, Beijing) were used for polymerase chain reaction (PCR). The amplification of DNA was done by using Mastercycler® Nexus (Eppendorf North America, Inc.). PCR amplifications were involved 1 cycle of initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s (denaturation), 48°C for 30 s (annealing) and 72°C for 1 min (extension), with a final extension at 72°C for 10 min. Ultra-pure water was used as negative

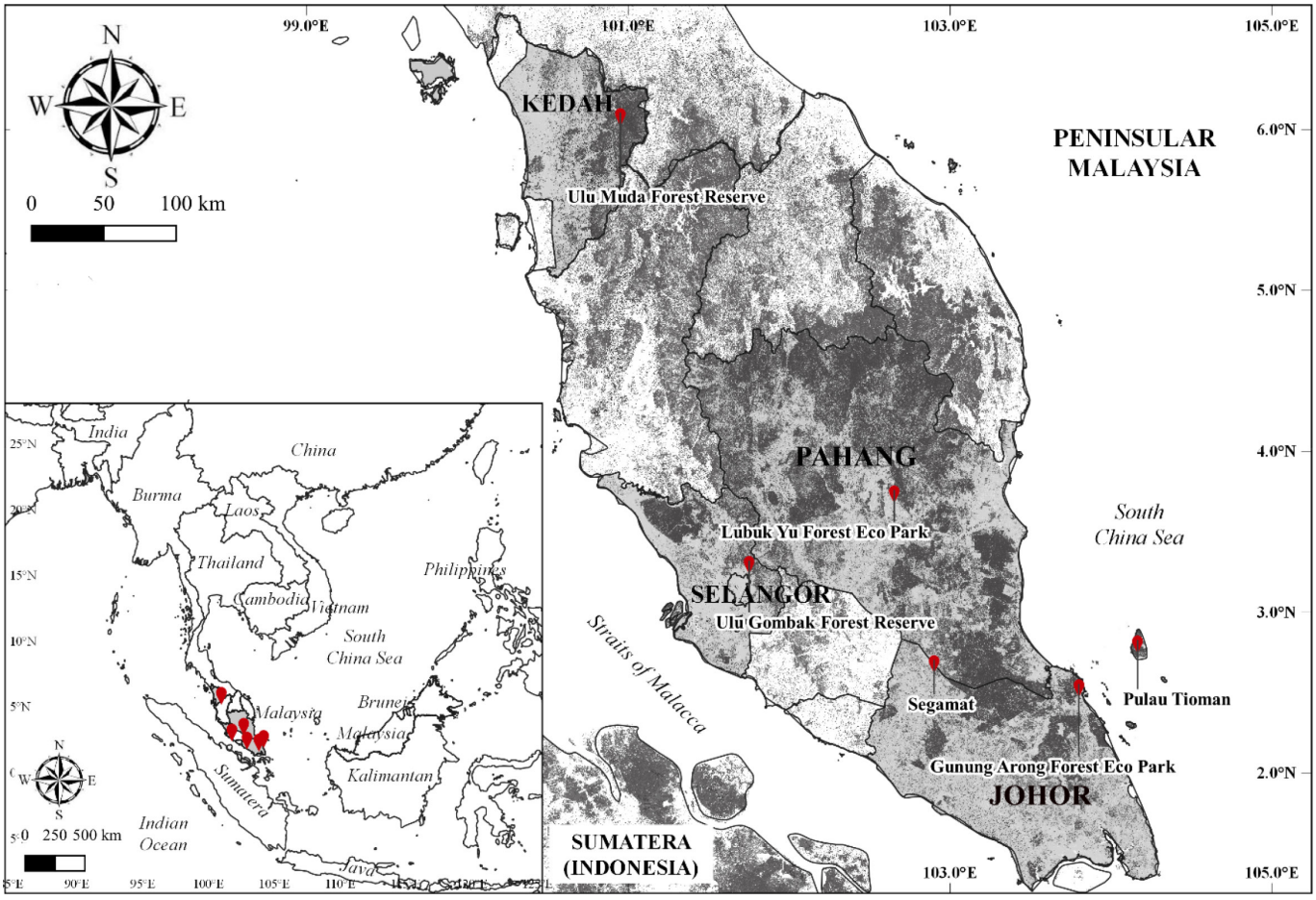

Figure 1. A map of study areas in the states of Johor, Pahang, Selangor, and Kedah in Peninsular Malaysia (top right), the locations where wild rats are captured and marked with red pins and labelled with the locality names (QGIS version 3.10.14-A Coruña).

Table 1. Localities and sample size from which wild rats were collected in several regions in Peninsular Malaysia

Localities	Forest type	Latitude	Longitude	Samples size (n)
Segamat, Johor	Modified forest (i.e., immature, young-mature, and matured oil palm plantations)	2° 42′ 0.813″ N	102° 54′ 0.8274″ E	65
Gunung Arong Forest Eco Park, Johor	Lowland dipterocarp forest (recreational forest with 273m)	2° 33′ 0.6762″ N	103° 48′ 0.4788″ E	7
Pulau Tioman, Pahang	Forest island (Ecotourism Island and recreational forest with 200m)	2° 49′ 21.9426″ N	104° 9′ 49.9752″ E	12
Lubuk Yu Forest Eco Park, Pahang	Lowland dipterocarp forest (recreational forest with waterfall)	3° 45′ 0.4356″ N	102° 39′ 0.0498″ E	11
Ulu Muda Forest Reserve, Kedah	Primary lowland dipterocarp forest (forest reserve)	6° 6′ 0.8892″ N	100° 57′ 0.7884″ E	23
Ulu Gombak Forest Reserve, Selangor	Secondary lowland dipterocarp forest (forest reserve with logging activities)	3° 19′ 0.5046″ N	101° 45′ 0.129″ E	6

Table 2. List of primers for amplification of 18SrDNA and COX1 genes, with primers and sequences as published, as well as fragment length and annealing temperature in polymerase chain reactions (PCR)

Genes	Primers	Sequence (5′ - 3′)	Length (bp)	°C	References
18SrDNA	18S cestode F 18S cestode R	TAATGGAATAGGACTTCGGT ATGACGCCAATCCAAGGA	1202	48	This study
COX1	pan cestode COX1 82F pan cestode COX1 209R	TGGGTTATTGTTTGCTATGTTTTCWA CCCCTATTATCATAGTAACMGAACTAAA	206	48	Poon *et al*. (2017)

control for each PCR reaction. After the PCR reactions, 4 µl of each PCR product and 4 µl of 100 base pair (bp) PCR ladder was loaded into the wells of 2% agarose gels by TAE electrophoresis. The gel electrophoresis was run for about 35 min in 1× TAE buffer at 80 V and 180 mA. Then, the gels were visualized under an ultra-violet (UV) illuminator machine.

DNA Sequence and phylogenetic analysis

All sequences were edited using the BioEdit programs (version 7.2.5). Then, the edited sequences were aligned accordingly using the CLUSTALW multiple alignment program in Molecular Evolutionary Genetics Analysis Version X (MEGA X) (Kumar *et al*., 2018). To infer the phylogenetic relationships in a wider perspective, several species of cestodes were downloaded from GenBank included in this study (Table 3).

Phylogenetic trees were constructed using maximum-likelihood (ML) method and neighbour-joining (NJ) method analysis for 18SrDNA and COX1 data sequences using MEGA X software. The phylogeny bootstrap test is with 1 000 bootstrap replications by using the best fit substitution models such as Kimura 2-parameter (K2) and evolutionary rates among site (+G = discrete gamma distribution) for 18SrDNA and COX1, respectively.

The haplotype sequences were calculated from the 18SrDNA and COX1 sequences datasets using DNA Sequence Polymorphism (DnaSP) software (version 6.12.03) (Rozas *et al*., 2017). NETWORK (10.2.0.0) was used to build a minimum-spanning network (MSN) of haplotypes for cestode in this study to obtain their haplotype relationships (Brandelt *et al*., 1999). The haplotype network was constructed with a median-joining algorithm (Brandelt *et al*., 1999).

RESULTS

Occurrence of cestode infection in wild rodents

A total of 124 wild rats were successfully captured and screened for cestode parasites resulted with an overall prevalence of 11.29% (n = 40) with *Hymenolepis diminuta* (77.5%, n = 31) followed by *Hydatigera parva* (15%, n = 6) and *Hydatigera taeniaeformis* (7.5%, n = 3).

Majority of the *Hymenolepis diminuta* infections were recovered from 3 rat species (*Leopoldamys sabanus*, *Maxomys surifer*, and *Rattus tiomanicus*) while *Hydatigera* species were recovered from *R. argentiventer* and *R. tiomanicus*. *Hydatigera taeniaeformis* was the lesser cestode infecting only *R. tiomanicus*. Prevalence of infection according to the host showed *R. tiomanicus* (64.29%) with the highest infections followed by *L. sabanus* (21.43%) (Table 3).

Phylogenetic relationships

The 18SrDNA and COX1 amplicons of *H. diminuta*, *Hy. parva* and *Hy. taeniaeformis* were sequenced, annotated, and deposited in GenBank (Table 3). All the sequences were amplified with total length 205 and 1202 base pairs (bp), respectively. Sequences obtained from both *H. diminuta* and *Hy. parva* represents the first record in Malaysia. A total of four sequences of the 18SrDNA and five sequences of the COX1 were accessed from the GenBank and compared with the 15 sequences obtained from in this study. All the GenBank accession numbers were also listed in the Table 3. The alignment of the 18SrDNA data set (1202 bp) consisted of 4 conserved and 1197 variable sites, of which 1144 parsimony-

Table 3. List of GenBank accession number for cestode species based on 18SrDNA and COX1 sequences used in this study, along with some information regarding their host species, locality, and references

Family	Cestode species	Host		Localities	Accession number		Reference
					18SrDNA	COX1	
Diphyllobothriidae	*D. stemmacephalum*	*Homo sapiens*	Human	Japan	NA	LC090628	Yamasaki *et al*. (2016)
	D. stemmacephalum	*Lagenorhynchus acutus*	Atlantic white-sided dolphin	NA	AF124459	NA	Olson & Caira (1999)
Hymenolepididae	*H. diminuta*	*Rattus tiomanicus*	Malayan field rat	Lubuk Yu Pahang, MY	OK104126	MZ905480	This study
	H. diminuta	*Leopoldamys sabanus*	Long-tailed giant rat	Pulau Tioman Pahang, MY	OK104127	MZ905481	This study
	H. diminuta	*Rattus tiomanicus*	Malayan field rat	Lubuk Yu Pahang, MY	OK104128	MZ905482	This study
	H. diminuta	*Rattus tiomanicus*	Malayan field rat	Segamat Johor, MY	OK104129	MZ905483	This study
	H. diminuta	*Rattus tiomanicus*	Malayan field rat	Pulau Tioman Pahang,	OK104130	MZ905484	This study
	H. diminuta	*Maxomys surifer*	Red spiny rat	Pulau Tioman Pahang, MY	OK104131	MZ905485	This study
	H. diminuta	*Leopoldamys sabanus*	Long-tailed giant rat	Pulau Tioman Pahang, MY	OK104132	MZ905486	This study
	H. diminuta	*Leopoldamys sabanus*	Long-tailed giant rat	Pulau Tioman Pahang, MY	OK104133	MZ905487	This study
	H. diminuta	*Rattus norvegicus*	Brown rat	India	NA	KJ576905	NA
	H. diminuta	*Rattus norvegicus*	Norway rat	Poland	JX310720	NA	Tanaka *et al*. (2014)
Taeniidae	*Hy. taeniaeformis*	*Rattus tiomanicus*	Malayan field rat	Segamat Johor, MY	OK104116	MZ905470	This study
	Hy. taeniaeformis	*Rattus tiomanicus*	Malayan field rat	Ulu Muda Kedah, MY	OK104117	MZ905471	This study
	Hy. taeniaeformis	*Rattus rattus*	Black rat	Malaysia	NA	LC223129	Okamoto *et al*. (1995)
	Hy. taeniaeformis	*Rattus norvegicus*	Brown rat	China	NA	MF380376	Zhao *et al*. (2020)
	Hy. taeniaeformis	NA	NA	Japan	AB731629	NA	Nakao *et al*. (2013)
	Hy parva	*Rattus tiomanicus*	Malayan field rat	Segamat Johor, MY	OK104134	MZ905488	This study
	Hy. parva	*Rattus tiomanicus*	Malayan field rat	Segamat Johor, MY	OK104135	MZ905489	This study
	Hy. parva	*Rattus tiomanicus*	Malayan field rat	Segamat Johor, MY	OK104136	MZ905490	This study
	Hy. parva	*Rattus argentiventer*	Ricefield rat	Segamat Johor, MY	OK104137	MZ905491	This study
	Hy. parva	*Rattus tiomanicus*	Malayan field rat	Segamat Johor, MY	OK104138	MZ905492	This study
	Hy. parva	*Apodemus sylvaticus*	Wood mouse	Spain	NA	EU544580	Lavikainen *et al*. (2008)
	Hy. parva	NA	NA	Spain	AB731627	NA	Nakao *et al*. (2013)

Note: N/A = Not Applicable; MY = Malaysia.

informative; while the alignment of the COX1 data set (205 bp) consisted of 76 conserved and 128 variable sites, of which 106 parsimony-informative.

We constructed the ML and NJ phylogenetic trees using the Kimura 2-parameter model with gamma distribution (K2 + G) and 1000 bootstrap replicates for both 18SrDNA (Figure 2) and COX1 (Figure 3). The 18SrDNA and COX1 gene fragments clearly demonstrated *H. diminuta* (e.g., Pulau Tioman, Lubuk Yu, Segamat and one sample from Poland) formed a single monophyletic group. The bootstrap values for 18SrDNA gene were strongly supported in the ML (98%) and NJ (100%) analyses. Meanwhile, the COX1 gene results showed similar topology despite lower bootstrap values for the ML (72%) and (69%) trees.

Hydatigera parva and *Hy. taeniaeformis*, also formed its own monophyletic group in 18SrDNA analyses. Bootstrap values for *Hy. taeniaeformis* from 18SrDNA and COX1 genes were highly supported (> 90%) with *Hy. taeniaeformis* from Japan (AB731629), China (MF380376) and Malaysia (LC223129). *Hydatigera parva* from Spain (AB731627) is polyphyletic with *Hy. parva* from this study based on the 18SrDNA phylogenetic tree. Unlike, for the COX1 gene, *Hy. parva* sequences from this study diverted out from the *Hydatigera* branch and possibly due to the short sequence size (205 bp) (Figure 3).

Genetic divergence and diversity

Genetic relationships of the cyclophyllid worms were analysed using K2P distance model with reference to the 18SrDNA and COX1 mitochondrial genes sequences. The genetic divergence obtained from both genes were summarized by the mean percentage pairwise K2P distance (%) and standard error of means (± SE) in Tables 5 and 6. Analyses were conducted to further clarify the polyphyletic clade obtained from the phylogenetic relationship tree.

Intra- and inter-species cestode levels were determined through a study conducted by Galimberti *et al*. (2012) and Zhang *et al*. (2014) on the family Taeniidae specifically on the species *Taenia*. Their study referred to a COX1 marker that had a length ranging from 380 to 444 bp. According to Zhang *et al.* (2014), the mean percentage of K2P genetic divergence of 0.71 ± 0.17% represents intraspecific variation, while 15.97 ± 0.22% and higher distances is considered interspecific variation. This study adopted estimates by Galimberti *et al*. (2012), whereby 2.0% and higher percentage of mean K2P distance was considered as optimal barcoding threshold (OT) values.

All the Malaysian isolate *H. diminuta*, *Hy. parva* and *Hy. taeniaeformis*, showed no significant difference in the divergence value (< 2%) between the same species and region in this study. However, there are differences between regions as shown in Table 4 and 5.

Our 18SrDNA results showed high mean intraspecific divergence between the Malaysian and Polish *H. diminuta* (12.79 ± 0.91%) (Table 4). Nevertheless, based on COX1 gene, there was a lower mean intraspecific value of 4.39 ± 2.51% between *H. diminuta* from Malaysia and India (Table 5). However, *H. diminuta* from Malaysia and outgroup (AF124459) was highly distinct with inter-specific divergence value of 29.79 ± 2.13% while *H. diminuta* from Malaysia and outgroup (LC090628) was 17.36 ± 5.87% for 18SrDNA and COX1 genes, respectively.

Besides *H. diminuta*, *Hy. parva* showed the highest K2P divergence (55.07 ± 4.59%) and 24.31 ± 7.82%) with others *Hy. parva* in COX1 and 18SrDNA genes respectively. A very significant difference when compared to *Hy. taeniaeformis* (see Table 4 and 5).

DNA single polymorphism analysis was conducted on selected cestode sequences based on the 18SrDNA and COX1 gene. For the 18SrDNA gene, eighteen haplotypes were found in 18 taxa, giving a haplotype diversity (Hd) value of 1.000 and nucleotide diversity (π) value of 0.272 while ten haplotypes for COX1 in 19 taxa, giving a haplotype diversity (Hd) value of 0.871 and nucleotide diversity (π) value of 0.090. The neutrality test showed that there were not significantly different between the Tajima's D (-0.13136 and -0.43206) (P > 0.10) Fu and Li's D* (0.48463 and -0.87961)

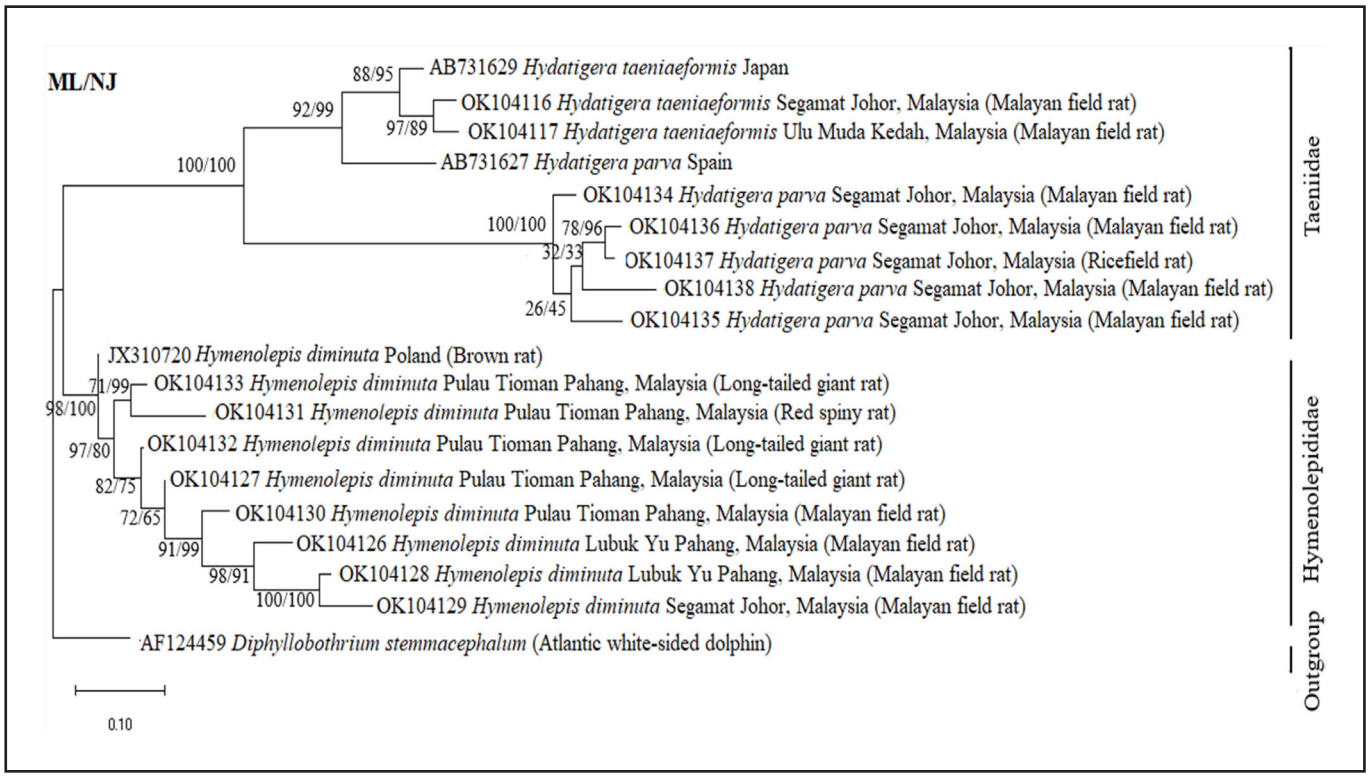

Figure 2. Phylogenetic analysis of *Hydatigera* species and *Hymenolepis* species based on 1202 bp 18SrDNA gene sequences. The tree was inferred using both maximum-likelihood (ML) method and neighbour-joining (NJ) method estimated via the Kimura 2-parameter with gamma distribution (K2P + G) and 1,000 bootstrap replications in MEGAX.

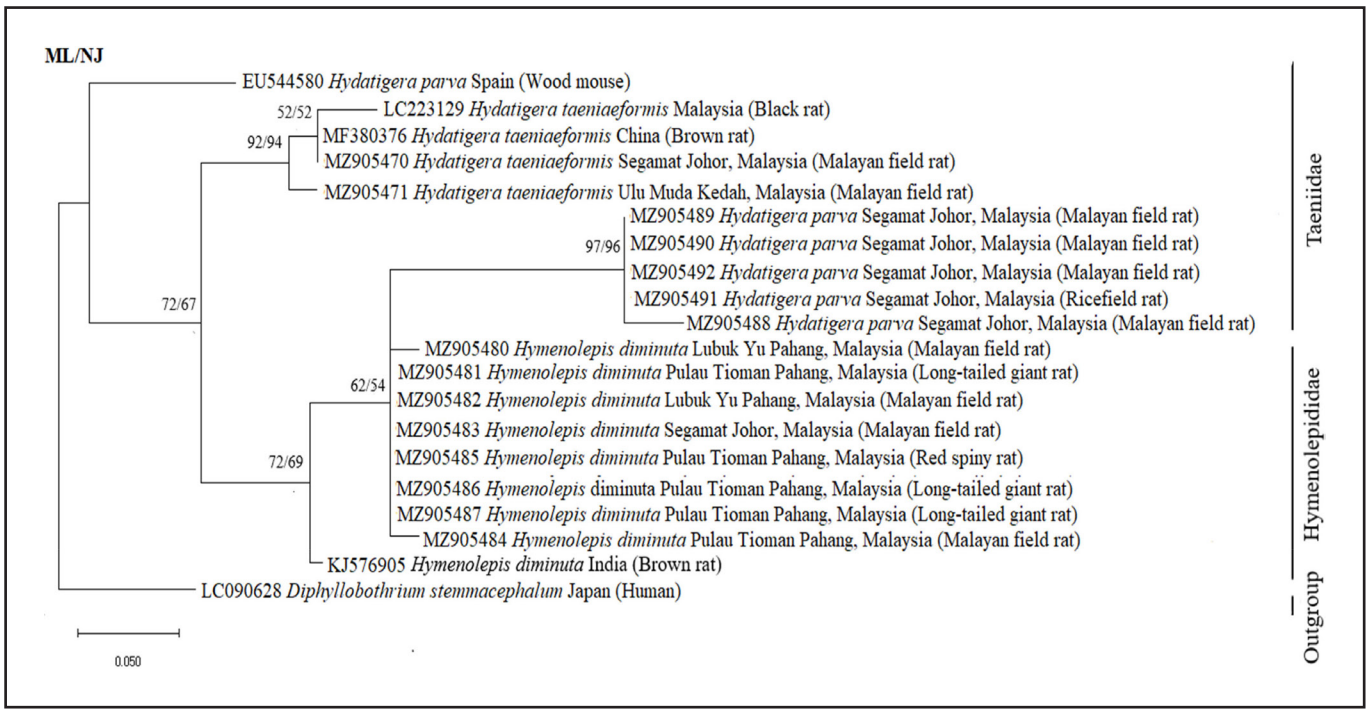

Figure 3. Phylogenetic analysis of *Hydatigera* species and *Hymenolepis* species based on 205 bp COX1 gene sequences. The tree was inferred using both maximum-likelihood method (ML) and neighbour-joining method (NJ) estimated via the Kimura 2-parameter with gamma distribution (K2P + G) and 1,000 bootstrap replications in MEGAX.

Table 4. Mean percentage (%) of pairwise genetic divergence based on the Kimura 2-parameter model (K2P) are below the diagonal, and their standard errors of means (±SE) are given above the diagonal among *Hymenolepis diminuta* and *Hydatigera* spp., namely as *Hymenolepis diminuta* Malaysia: *Hdim* MY; *Hydatigera parva* Malaysia: *Hypa* MY; *Hydatigera taeniaeformis* Malaysia: *Hyta* MY and three sequence obtained from GenBank (*H. diminuta* Poland: *Hd*im PL; *Hy. parva* Spain: *Hypa* ES; *Hy. taeniaeformis* Japan: *Hyta* JP) and *Diphyllobothrium stemmacephalum: Ds* (AF124459) as outgroup of targeted 18SrDNA gene

		1	2	3	4	5	6	7
1	*Hdim* MY		0.91	6.35	5.13	4.53	4.65	2.13
2	*Hd*im PL	12.79		5.34	4.15	3.75	3.73	1.57
3	*Hypa* MY	79.88	62.41		4.59	4.56	4.80	5.91
4	*Hypa* ES	65.33	46.83	55.07		2.04	1.78	4.42
5	*Hyta* MY	59.95	43.03	58.43	22.89		1.03	4.17
6	*Hyta* JP	61.65	43.48	59.25	19.12	9.39		4.07
7	*Ds* (Outgroup)	29.79	14.35	70.24	49.69	50.04	48.45	

Table 5. Mean percentage (%) of pairwise genetic divergence based on the Kimura 2-parameter model (K2P) are below the diagonal, and their standard errors of means (±SE) are given above the diagonal among *Hymenolepis diminuta* and *Hydatigera* spp., namely as *Hymenolepis diminuta* Malaysia: *Hdim* MY; *Hydatigera parva* Malaysia: *Hypa* MY; *Hydatigera taeniaeformis* Malaysia: *Hyta* MY and four sequence obtained from GenBank (*H. diminuta* India: *Hdim* IN; *Hy. parva* Spain: *Hypa* ES; *Hy. taeniaeformis* MALAYSIA (LC223129): *Hyta* MY (LC223129); *Hy. taeniaeformis* China: *Hyta* CN) and *Diphyllobothrium stemmacephalum: Ds* (LC090628) as outgroup of targeted COX1 gene

		1	2	3	4	5	6	7	8
1	*Hdim* MY		2.51	4.36	6.09	4.88	5.06	5.23	5.87
2	*Hdim* IN	4.39		5.70	5.45	3.96	4.59	4.28	6.01
3	*Hypa* MY	11.24	15.63		7.82	7.34	7.44	7.71	6.26
4	*Hypa* ES	17.36	15.23	24.31		5.76	5.49	6.19	5.38
5	*Hyta* MY	13.04	9.26	22.41	16.34		2.04	0.68	5.58
6	*Hyta* MY (LC223129)	13.88	11.71	23.66	15.25	3.33		1.86	5.95
7	*Hyta* CN	13.93	10.06	23.28	17.33	0.65	2.62		5.93
8	*Ds* (Outgroup)	17.36	17.10	19.09	13.43	16.17	17.12	17.10	

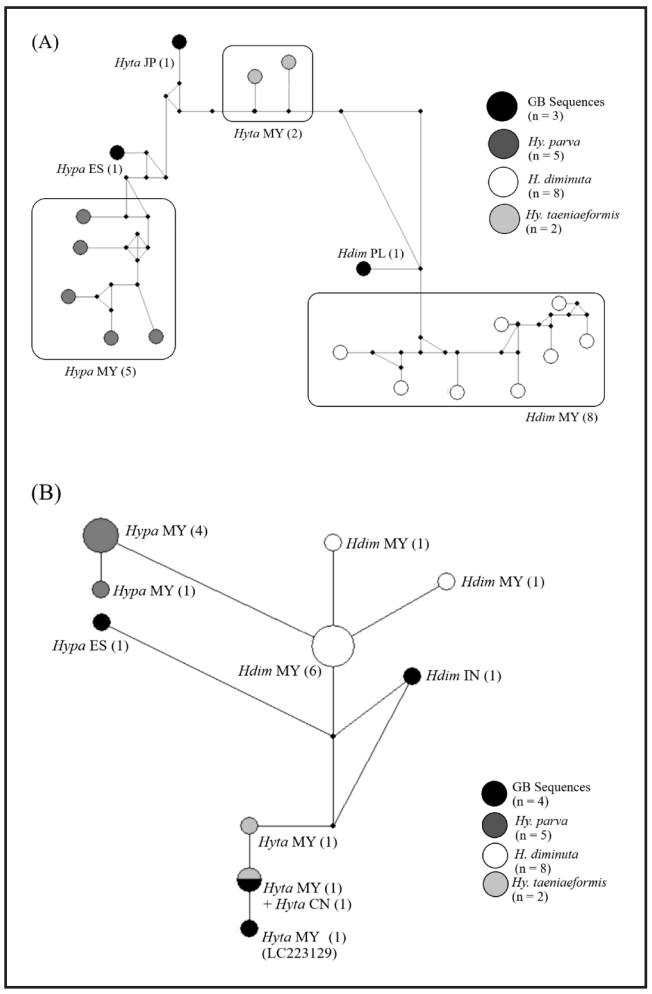

Figure 4. Minimum-spanning network of cestodes inferred from (A) 18SrDNA and (B) COX1 haplotypes using NETWORK 10.2.0.0. The size of the circle represents the number of individuals per haplotype (18 taxa and 18 haplotypes) and (19 taxa and 10 haplotypes) for 18SrDNA and COX1, respectively. Notes: MY (Malaysia); CN (China); ES (Spain); JP (Japan); PL (Poland); *Hdim* (*H. diminuta*); *Hypa* (*Hy. parva*); *Hyta* (*Hy. taeniaeformis*).

(P > 0.10) and Fu and Li's F* (0.35171 and -0.86953) (P > 0.10) for both 18SrDNA and COX1, respectively.

From the haplotype data, the minimum-spanning network (MSN) was constructed to illustrate the relationships between the three cestode species. The median joining network for 18SrDNA and COX1 were significant with the phylogenetic constructions (Figure 2 and 3). For 18SrDNA, all taxa from *H. diminuta*, *Hy. taeniaeformis* and *Hy. parva* constructed its own haplotypes without any information shared between individuals as shown in Figure 4A.

However, for a COX1, *H. diminuta* from Malaysia shared a single haplotype with six individuals namely *Hdim* MY (6) as well as *Hy. taeniaeformis* from Malaysia shared the same haplotype with *Hy. taeniaeformis* from China (*Hyta* MY (1) + *Hyta* CN (1)) and linked to *Hy. taeniaeformis* Malaysia (LC223129). For COX1 gene, four *Hy. parva* taxa from Malaysia shared a single haplotype, with only one taxon separated (Figure 4B).

DISCUSSION

The advent of molecular phylogenetic studies has largely not been able to resolve many interrelationships of cestode worms in rats but relatively useful for species identification. The task is made more difficult with only a limited number of records in Malaysia (Okamoto *et al*., 1995; Lavikainen *et al*., 2016) and in the Southeast Asia region (Butboonchoo *et al*., 2016; Sanpool *et al*., 2017; Catalano *et al*., 2018; Alvi *et al*., 2021). Therefore, molecular descriptions of three cestodes parasites from rodents (*H. diminuta*, *Hy. taeniaeformis* and *Hy. parva*) were analysed with reference to the phylogenetic relationships, genetic divergence, and haplotype networking. Sequence obtained from both *H. diminuta* and *Hy. parva* represents the first records in Malaysia. In this study, 18SrDNA was shown to be a better marker to resolve phylogenetic relationships in different species, including high taxonomic levels of parasitic platyhelminthes such as cestodes (Mariaux, 1996; Foronda *et al*., 2004; Yan *et al*., 2013; Tanaka *et al*., 2014).

Hymenolepis diminuta is a generalist parasite and commonly found in various animals' taxa, including humans (Kan *et al*., 1981; Tena *et al*., 1998; Marangi *et al*., 2003; Rohela *et al*., 2003; Panti-May *et al*., 2020). The phylogenetic analysis in this study placed *H. diminuta* into species level with all the branches clustered together with the rest particularly with the Polish and Indian isolates indicating that genetic characteristics are not always related to geographical distribution (Mohammadzadeh *et al*., 2007; Yang *et al*., 2017).

However, the Malaysian *Hy. parva* isolate appeared to be genetically distinct from European *Hy. parva* (Spain), but closely related to the local isolates. Unfortunately, previous molecular, biogeography and even ecological records is limited among the family Taeniidae (Lavikainen *et al*., 2008; Nakao *et al*., 2013; Lavikainen *et al*., 2016; Catalona *et al*., 2018) therefore taxonomic status remains unclear (Lavikainen *et al*., 2016). The crucial information in separating parasite group into species is based in their allopatric or sympatric occurrences, reproductive isolation, and host specialisation (Hoberg, 2006).

Previously classified from the genus of *Taenia*, *Hy. taeniaeformis* is among the most studies parasite due to their wide distribution and the existence of three morphologically cryptic species (Lavikainen *et al*., 2016; Alvi *et al*., 2021). In this study, *Hy. taeniaeformis* showed close relationship with the Chinese and Japanese isolates for both 18SrDNA and COX1 genetic markers despite their species complexity. The latest studies by Alvi *et al*. (2021) and Mulinge *et al*. (2020), recorded *Hy. taeniaeformis* infection in *R. rattus* in Pakistan and Malaysia (Nakao *et al*., 2013). Lavikainen *et al*. (2016) described *Hy. taeniaeformis* formed distinct clades with different hosts such as the Cricetidae and Felidae families.

As previously stated, molecular characterization of cestode worms is still not well established yet. Therefore, more work is required to clarify interrelationships status between *Hydatigera* spp. due to the lack of universal criteria in distinguishing inter-specific and intra-specific genetic variants between species complex among Taeniidae group (Galimberti *et al*., 2012; Zhang *et al*., 2014; Lavikainen *et al*., 2016). A high genetic heterogeneity was shown among *Hy. taeniaeformis* isolates which was possibly to be due to host's (rat) movement in the past 300 years across the world (Wilson & Reeder, 2005) as exhibited in its widespread presence in Asia, Australia, Africa, and Europe (Lavikainen *et al*., 2016).

CONCLUSION

Molecular characterization of cestode worms from wild rats is not well established. Therefore, utilizing 18SrDNA gene proved to be a useful marker for the identification and clarification of the phylogenetic relationships between *Hymenolepis diminuta*, *Hydatigera parva*, and *Hydatigera taeniaeformis* (syn *Taenia taeniaeformis*). Of the three species studied, *H. diminuta* lineage was clearly distinct while *Hydatigera* spp., on the other hand, was among the complex species. The 18SrDNA gene sequence also provided reliable data to distinguish the lineages of various cestode populations due to genetically high conservation and strict maternal inheritance. Therefore, future research on molecular systematics

and taxonomy in the order of Cyclophyllidean cestodes is important to further unravel linkages particular between complex species.

Conflict of Interest

There are no conflicts of interest declared by the authors.

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