Current Research in Parasitology & Vector-Borne Diseases 5 (2024) 100154

Contents lists available at ScienceDirect

Current Research in Parasitology & Vector-Borne Diseases

journal homepage: www.sciencedirect.com/journal/current-research-in-parasitology-and-vector-borne-diseases

Redescription, molecular characterisation and *Wolbachia* endosymbionts of *Mansonella* (*Tupainema*) *dunni* (Mullin & Orihel, 1972) (Spirurida: Onchocercidae) from the common treeshrew *Tupaia glis* Diard & Duvaucel (Mammalia: Scandentia) in Peninsular Malaysia

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ARTICLE INFO

Keywords:
Filaria
Host switching
Mansonella (*Tupainema*) *dunni*
Mansonella (*Mansonella*) *ozzardi*
Multi-locus sequence analysis
Onchocercinae
Wolbachia
Southeast Asia

ABSTRACT

The genus *Mansonella* Faust, 1929 includes 29 species, mainly parasites of platyrrhine monkeys in South America and anthropoid apes in Africa. In Malaysia, *Mansonella* (*Tupainema*) *dunni* (Mullin & Orihel, 1972) was described from the common treeshrew *Tupaia glis* Diard & Duvaucel (Scandentia). In a recent classification of the genus *Mansonella*, seven subgenera were proposed, with *M.* (*Tup.*) *dunni* as a monotypic species in the subgenus *Tupainema*. In this study, we collected new material of *M.* (*Tup.*) *dunni* from common treeshrews in Peninsular Malaysia and redescribed the morphological features of this species. We found that *M.* (*Tup.*) *dunni* differs from *M.* (*Cutifilaria*) *perforata* Uni et al., 2004 from sika deer *Cervus nippon* (Cetartiodactyla) in Japan, with regards to morphological features and predilection sites in their respective hosts. Based on multi-locus sequence analyses, we examined the molecular phylogeny of *M.* (*Tup.*) *dunni* and its *Wolbachia* genotype. Species of the genus *Mansonella* grouped monophyletically in clade ONC5 and *M.* (*Tup.*) *dunni* was placed in the most derived position within this genus. *Mansonella* (*Tup.*) *dunni* was closely related to *M.* (*M.*) *ozzardi* (Manson, 1897) from humans in Central and South America, and most distant from *M.* (*C.*) *perforata*. The calculated p-distances between the *cox*1 gene sequences for *M.* (*Tup.*) *dunni* and its congeners were 13.09% for *M.* (*M.*) *ozzardi* and 15.6–16.15% for *M.* (*C.*) *perforata*. The molecular phylogeny of *Mansonella* spp. thus corroborates their morphological differences. We determined that *M.* (*Tup.*) *dunni* harbours *Wolbachia* endosymbionts of the supergroup F genotype, in keeping with all other *Mansonella* species screened to date.

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https://doi.org/10.1016/j.crpvbd.2023.100154
Received 11 August 2023; Received in revised form 5 November 2023; Accepted 11 November 2023
Available online 23 November 2023

1. Introduction

Mansonella Faust, 1929 is one of 90 genera of the family Onchocercidae (Spirurida) and currently includes 29 species, one subspecies and one *species inquirenda* (Orihel and Eberhard, 1982; Anderson, 2000; Bain, 2002; Gibbons, 2010; Bain et al., 2014, 2015). Adult worms of *Mansonella* spp. occur in the subcutaneous connective tissues, body cavity and dermis of primates, including humans, Sciuridae and Caviidae (Rodentia), Procyonidae and Ursidae (Carnivora), Cervidae (Cetartiodactyla) and Tupaiidae (Scandentia). Unsheathed microfilariae occur in the blood or skin and develop in the thoracic muscles of haematophagous dipterans of the genera *Culicoides* (Ceratopogonidae) and *Simulium* (Simuliidae) (Eberhard and Orihel, 1984; Anderson, 2000).

Three species of the genus *Mansonella*, *M.* (*Esslingeria*) *perstans* (Manson, 1891), *M.* (*Mansonella*) *ozzardi* (Manson, 1897) and *M.* (*E.*) *streptocerca* (Macfie & Corson, 1922), cause human mansonellosis. *Mansonella* (*E.*) *perstans* is widely distributed in sub-Saharan Africa, parts of Central and South America as well as in the Caribbean. It is estimated that 114 million people are infected with this parasite in wet subtropical and tropical areas of Africa (Simonsen et al., 2011; Mediannikov and Ranque, 2018; Ta-Tang et al., 2018). *Mansonella* (*M.*) *ozzardi* is distributed in parts of Central America, Amazonia, and the Caribbean, whereas *Mansonella* (*E.*) *streptocerca* is endemic in parts of Central and West Africa. Humans are the only known natural vertebrate host for *M.* (*M.*) *ozzardi*, but primates other than humans can serve also as additional natural hosts for *M.* (*E.*) *perstans* and *M.* (*E.*) *streptocerca*. Furthermore, *M.* (*E.*) *rodhaini* (Peel & Chardome, 1946), whose natural host is the bonobo *Pan paniscus* Schwarz (Primates, Hominidae), is reported to cause human infections in Gabon (Richard-Lenoble et al., 1982, 1988; Orihel and Eberhard, 1998). *Sandnema digitatum* (Chandler, 1929) [as *Mansonella digitatum* (Chandler, 1929)] was collected from a male patient in Thailand (Sucharit, 1988). These findings suggest that zoonotic cases in humans caused by *Mansonella* species and closely related genera are not uncommon.

Eberhard and Orihel (1984) proposed a new classification of the genus *Mansonella* (syn. *Tetrapetalonema* Faust, 1935) that recognized 25 species in five subgenera and considered *M.* (*T.*) *zakii* (Nagaty, 1935) a *species inquirenda*. Recently, Bain et al. (2015), retaining *M.* (*T.*) *zakii* as *species inquirenda*, validated 29 species in seven subgenera in the genus *Mansonella* based on morphological characteristics: *Mansonella* Faust, 1929; *Cutifilaria* Bain & Schulz-Key, 1974; *Esslingeria* Chabaud & Bain, 1976; *Filyamagutia* Bain & Uni, 2015; *Pseudolitomosa* Yamaguti, 1941; *Tetrapetalonema* Faust, 1935; *Tupainema* Eberhard & Orihel, 1984.

In both these classifications, *M.* (*Tupainema*) *dunni*, described as *Tetrapetalonema dunni* from the common treeshrew *Tupaia glis* Diard & Duvaucel (Tupaiidae) by Mullin and Orihel (1972), was listed as monotypic species in the subgenus *Tupainema*, based on its morphological characteristics, the uniqueness of its host and its distribution in Southeast Asia. According to Chabaud and Bain (1994) and Bain (2002), the *Mansonella* lineage has arisen from *Sandnema sunci* (Sandground, 1933) from Eulipotyphla (as Insectivora) or forms of the subgenus *Tupainema* in Southeast Asia. To elucidate the phylogeny of *M.* (*Tup.*) *dunni*, we examined its morphological features, molecular characteristics and *Wolbachia* endosymbiont genotype.

Molecular studies of *Mansonella* spp. have been conducted mainly on *M.* (*M.*) *ozzardi* and *M.* (*E.*) *perstans* from humans (Crainey et al., 2018; Poole et al., 2019; Sandri et al., 2021), including recently the report of their whole genomes (Sinha et al., 2023b). The *Wolbachia* genomes, *w*Mpe from *M.* (*E.*) *perstans* and *w*Moz from *M.* (*M.*) *ozzardi*, have also recently been published (Sinha et al., 2023a). In addition, very few gene sequences of *M.* (*Tet.*) *mariae* Petit et al., 1985 from *Saimiri sciureus* L. (Primates: Cebidae) from Guyana, *M.* (*Tet.*) *atelensis amazonae* Bain & Guerrero, 2015 from *Cebus olivaceus* Schomburgk (Primates: Cebidae) in Venezuela (Bain et al., 2015) and *M.* (*C.*) *perforata* Uni et al., 2004 from sika deer *Cervus nippon* Temminck (Cetartiodactyla: Cervidae) in Japan are available (Ferri et al., 2011). The two species of *Mansonella* included in the phylogenetic study of Lefoulon et al. (2015), *M.* (*M.*) *ozzardi* and *M.* (*C.*) *perforata*, grouped in clade ONC5 of the family Onchocercidae.

A high diversity of filarial parasites has been recorded from vertebrates in Malaysia with 37 filarial species in 22 genera (Yen, 1983; Gibbons, 2010; Uni et al., 2017, 2020, 2022). However, *M.* (*Tup.*) *dunni* is so far the only species of *Mansonella* reported in Malaysia (Mullin and Orihel, 1972).

In this study, we examined the morphological features of *M.* (*Tup.*) *dunni* from common treeshrews in Peninsular Malaysia in detail and compared them to those of other subgenera within the genus. Based on multi-locus sequence analyses of seven genes (*12S* rDNA, *cox*1, *rbp*1, *hsp70*, *myoHC*, *18S* rDNA and *28S* rDNA), we determined its phylogenetic position within *Mansonella* and in relation to other members of the Onchocercidae. In addition, we identified the *Wolbachia* endosymbiont genotype of *M.* (*Tup.*) *dunni* using molecular analyses and compared it with those of other filariae.

2. Materials and methods

2.1. Collection of hosts and parasites

Between April 2015 and October 2016, we captured 45 common treeshrews from primary and secondary (second-growth) forests in five states in Peninsular Malaysia: 12 animals from Gunung Belumut Forest Eco Park (2.0666°N, 103.5255°E), Johor; 10 animals from Ulu Gombak Field Studies Centre (3.3249°N, 101.7534°E), Universiti Malaya, Selangor; 11 animals from Ulu Kenas Amenity Forest (4.6898°N, 100.8973°E), Perak; five animals from Perlis State Park, Wang Kelian (6.6979°N, 100.1927°E), Perlis; and seven animals from Jeram Linang Forest Eco Park (5.7433°N, 102.3737°E), Kelantan. All animals were captured using box cage traps baited with palm oil kernels or bananas. Adult filariae were collected from the subcutaneous connective tissues of the common treeshrews under a stereomicroscope and used for subsequent morphological and molecular analyses; thick blood smears were also examined.

2.2. Morphological methods

Isolated worms were fixed in 70% ethanol and temporarily mounted in lactophenol solution (R & M Chemicals, Essex, UK) for morphological examination under a compound microscope equipped with differential interference contrast. For each worm, we recorded body length and width, distance between the anterior extremity and nerve-ring, distance between the anterior extremity and vulva, and length of oesophagus, spicules and tail. We also recorded the length and width of microfilariae taken from the uteri of fixed adult females. Measurements of a representative female (J3 from Johor) and male (J4 from Johor) of *M.* (*Tup.*) *dunni* are presented first, followed by the range, including the representative specimens, in parentheses, and the mean in brackets. Measurements are in micrometres unless otherwise stated. The mid-region of a fixed female was embedded in paraffin, and sections were stained with haematoxylin and eosin (HE). Thick blood smears were stained with 3% Giemsa solution (pH 7.4) and examined for microfilariae under a compound microscope.

2.3. Molecular analysis of filarial nematodes

2.3.1. Multi-locus gene analyses

DNA was extracted from two females (ID nos. F1 and F3 from Perlis) using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). Samples were incubated at 56 °C with proteinase K for 4 h. Polymerase chain reaction (PCR) DNA amplification targeted partial sequences of seven genes: two mitochondrial genes, *12S* rDNA (*c.*450 bp) and *cox*1 (*c.*600 bp); and five nuclear genes, *18S* rDNA (*c.*740 bp), *28S* rDNA (*c.*900 bp), *MyoHC* (*c.*785 bp), *rbp*1 (*c.*640 bp) and *hsp70* (*c.*610 bp). PCR reactions were processed in a final volume of 20 µl (Casiraghi et al., 2001, 2004;

Lefoulon et al., 2015). Sequences of the amplified samples were obtained *via* Sanger sequencing (Eurofins Genomics). The produced chromatograms were analysed and cleaned using Chromas (http://www.technelysium.com.au/chromas.html).

2.3.2. Phylogenetic analysis

Sequences generated during the present study and those from the literature were concatenated to generate a dataset of 55 filarial species and a total of 3695 bp. Each locus was aligned independently with SATe-II (Liu et al., 2012). For each gene, the best-fitting substitution model was determined using the corrected version of the Akaike Information Criterion (AICc) in JModelTest v2.1.10 analyses (Guindon and Gascuel, 2003): TVM+Γ was the best fit for *12S* rDNA; TVMef+I+Γ for *18S* rDNA; TPM3+I+Γ for *28S* rDNA; TIM1+I+Γ for *cox*1; TN93+I+Γ for *hsp70*; TN93+I+Γ for *myoHC* and TPM3uf+Γ for *rbp*1. To root the trees, two species were included as outgroup: *Protospirura muricola* Gedoelst, 1916 (Spirurida: Spiruridae) and *Filaria latala* Chabaud & Mohammad, 1989 (Spirurida: Filariidae). The maximum-likelihood inference was executed by generating 10 random start trees with 1000 bootstrap replicates using RaxML-NG (Kozlov et al., 2019).

2.3.3. Additional molecular analyses

We determined partial sequences of the mitochondrial *cox*1 and *12S* rRNA genes of four females (ID nos. M1 and M2 from Johor; G3 and G4 from Selangor). DNA extraction, PCR amplification and sequencing were performed as described previously (Casiraghi et al., 2001; Agatsuma et al., 2005; Uni et al., 2017). We also cloned the PCR products of the ITS1 region into pGEM-T vectors and determined the sequences of the recombinant plasmid (Saijuntha et al., 2018). Phylogenetic trees of the nucleotide sequences of the *cox*1, *12S* rRNA genes and the ITS1 region of *M*. (*Tup*.) *dunni* were constructed using the maximum-likelihood method in MEGA7 with 1000 bootstrap replicates (Kumar et al., 2016). Sequence data were deposited in the GenBank database. The lengths of the sequence datasets used for the analyses were as follows: *cox*1, 393 bp; *12S* rDNA, 304 bp; and ITS1, 866 bp.

2.3.4. Genetic distances between filarial species

*Cox*1 sequences generated during the present study and those from the literature were analysed. First, the *cox*1 sequence divergence was estimated by the number of base differences per site between two sequences (uncorrected p-distance) using MEGA7 (Kumar et al., 2016). Subsequently, pairwise comparisons between the selected 17 *cox*1 sequences were processed, with each sequence representing a species.

2.4. Molecular analysis of Wolbachia

2.4.1. Wolbachia molecular screening

Wolbachia endosymbionts of *M.* (*Tup*.) *dunni* were determined by nested PCR screening of six genes (*16S* rDNA, *ftsZ*, *dnaA*, *coxA*, *fbpA* and *gatB*). Agarose gel electrophoresis was used to verify the successful amplification of PCR products. Amplicons were sequenced using the Sanger approach (Eurofins Genomics) and the resulting chromatograms analysed and cleaned using Chromas (http://www.technelysium.com.au/chromas.html).

2.4.2. Phylogenetic analyses of Wolbachia endosymbionts

Sequences generated during the present study and previously published from draft/complete genomes were aligned for each gene using SATe-II (Liu et al., 2012) and subsequently concatenated. The complete dataset comprised 53 *Wolbachia* genotypes and had a length of 2480 bp. For each gene, the best-fitting substitution model was determined using the corrected version of the Akaike Information Criterion (AICc), JModelTest analyses (v2.1.10) (Guindon and Gascuel, 2003). HKY+I was the best fit for *ftsZ*; HKY+I+Γ for *16S* rDNA; K81uf+Γ for *dnaA*; TMP3uf for *coxA*; and TMP3uf+Γ for *fbpA* and *gatB*. The phylogenetic relationships of the *Wolbachia* genotypes based on the concatenation of these six genes were inferred by the maximum-likelihood method using RaxML-ng (Kozlov et al., 2019). The dataset was partitioned to implement the best-fitting substitution model for each gene. The program was executed by generating 10 random start trees by using 1000 bootstrap replicates.

3. Results

3.1. Redescription of Mansonella (Tupainema) dunni (Mullin & Orihel, 1972) Eberhard & Orihel, 1984

3.1.1. Taxonomic summary

Host: *Tupaia glis* Diard & Duvaucel (Scandentia: Tupaiidae), common treeshrew.

Localities: (i) Gunung Belamut Forest Eco Park, Johor; (ii) Ulu Gombak Field Studies Centre, Universiti Malaya, Selangor; (iii) Ulu Kenas Amenity Forest, Perak; (iv) Perlis State Park, Wang Kelian, Perlis; and (v) Jeram Linang Forest Eco Park, Kelantan. Collection dates: (i) 24 April 2015; (ii) 19 October 2015; (iii) 27 September 2016; (iv) 5 October 2016; and (v) 29 February 2016, respectively.

Voucher material deposited: One female (MNHN-IN-110YT: UG2 from Ulu Gombak, Selangor) and one male (MNHN-IN-111YT: UG4 from Ulu Gombak, Selangor) were deposited in the Museum National d'Histoire Naturelle (MNHN), Paris, France. Additional specimens (7 females, MdF-1–7; 3 males, MdM-1–3) were deposited in the Museum of Zoology, Institute of Biological Sciences, Universiti Malaya.

Site in host: Adult worms occurred in the subcutaneous connective tissues of the neck and abdomen and microfilariae circulated in the blood of the common treeshrews.

Representative DNA sequences: All sequence data generated were deposited in the GenBank database. Sequence data for *M*. (*Tup*.) *dunni* (F1): *cox*1 (OR198850), *12S* rDNA (OR210206), *18S* rDNA (OR198492), *MyoHC* (OR555759), *hsp70* (OR555767), *rbp*1 (OR555760) and *28S* rDNA (OR206512) (Supplementary Table S1). Additional sequence data for specimens (M1-M2; G3-G4) of *M*. (*Tup*.) *dunni*: M1: *cox*1 (KY434306), *12S* rDNA (KY434310); M2: *cox*1 (KY434307), *12S* rDNA (KY434311); M2 C1: ITS1 (KY434312); M2 C2: ITS1 (KY434313); M2 C3: ITS1 (KY434314); G3: *cox*1 (KY434308); and G4: *cox*1 (KY434309). Sequence data for the *Wolbachia* endosymbionts of *M*. (*Tup*.) *dunni*: M1: *dnaA* (OR555761), *ftsZ* (OR555763) and *gatB* (OR555765); M2: *dnaA* (OR555762), *ftsZ* (OR555764) and *gatB* (OR555766) (Supplementary Table S2).

3.1.2. Morphological features of present specimens

General [Figs. 1 and 2]. Pre-oesophageal cuticular ring absent. Four external labial papillae and four cephalic papillae arranged in laterally elongated rectangle. Amphids lateral, approximately on level of external labial papillae (Figs. 1A and 2A). Anterior end slightly dilated, narrowing to conspicuous hemispherical prominence at apex in both sexes. Oesophagus not divided into muscular and glandular portions. Annular body swellings with coelomocytes in anterior half of body in both sexes. Caudal end of female with two lateral lappets and two terminal cones. In male, caudal papillae arranged in two ventrolateral groups near cloaca. Spicules unequal and dissimilar. Microfilaria without sheath.

Female. [Based on 10 gravid specimens from Johor, Selangor and Perak, Fig. 1A–E] Two to four annular body swellings with coelomocytes in anterior half of body: body swellings at 2 mm, 4 mm, 7 mm and 13 mm from anterior extremity in one female (body length, 45 mm). Transverse section of body slightly flattened dorsoventrally, cuticle thicker at lateral chords (Fig. 1D); lateral chords flat and wide; muscle cells high at dorsoventral sides. Body length 42 (31–45) [39] mm; width at midbody 150 (130–190) [151]. Nerve-ring 300 (158–310) [271] from anterior extremity. Oesophagus 608 (480–840) [667] long, slender. Vulva 685 (660–940) [746] from anterior extremity. Vulva located posterior to oesophago-intestinal junction in eight specimens (mean body length, 40 mm) (Fig. 1B), anterior to it in two specimens (body length 30–31 mm). Vagina uterina globular, its lumen composed of three

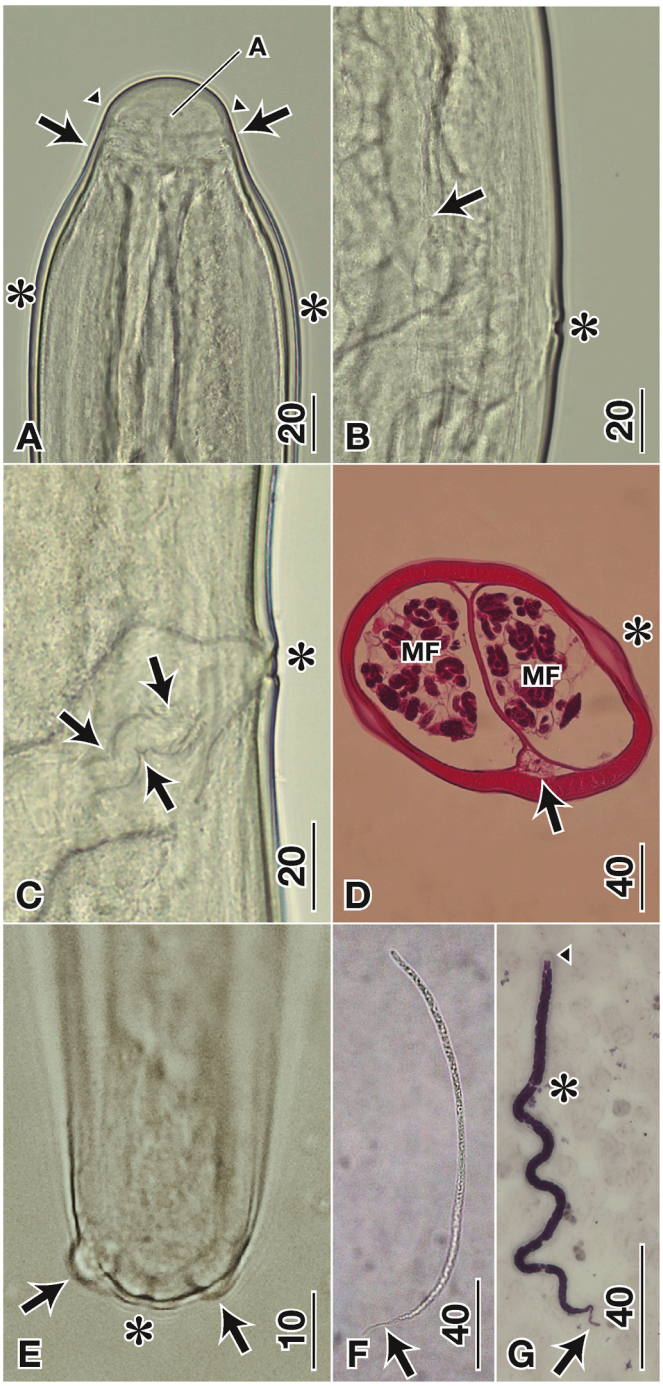

Fig. 1. Light micrographs of *Mansonella* (*Tupainema*) *dunni*. Females (**A-E**). Microfilariae (**F-G**). **A** Anterior end slightly dilated, with conspicuous hemispherical prominence at apex. Lateral view, showing amphid (*A*), labial papillae (*arrowheads*), cephalic papillae (*arrows*), and dilatation (*). **B** Lateral view showing oesophago-intestinal junction (*arrow*) and vulva opening (*). **C** Lateral view showing vulva opening (*) and globular vagina uterina and its bent lumen (*arrows*). **D** Transverse section of body showing two uteri including microfilariae (*MF*), intestine (*arrow*) and cuticular elevation (*) at lateral chords. HE staining. **E** Tail, dorso-ventral view, with two lateral lappets (*arrows*) and axial point divided at apex (*). **F** Microfilaria taken from the uteri of fixed female showing the anucleated area of the posterior end (*arrow*). **G** Microfilaria in Giemsa-stained thick blood smear, showing the cephalic space (*arrowhead*), nerve-ring (*), and anucleated area (*arrow*). Scale-bars are in micrometres.

to four bends (Fig. 1C). Uteri containing microfilariae. Tail bent ventrally, 203 (175–275) [232] long, with two lateral lappets and divided axial point (Fig. 1E).

Microfilaria. [Based on 10 specimens from anterior parts of uteri in 70% ethanol, Fig. 1F] Body 183–235 (mean, 203) long by 3–5 wide. [Based on 10 specimens in Giemsa-stained thick blood smears from a common treeshrew (No. 19), Fig. 1G] Body 147–188 (mean, 165) long by 3–4 wide. Cephalic space 3.2–3.8 long (1.9–2.3% of body length); nerve-ring 33–55 (20.2–30.6% of body length) from anterior extremity; anal pore 113–150 (77.2–84.1% of body length) from anterior extremity. Anucleate area of the posterior end 5–13 long (2.7–8.9% of body length).

Male [Based on 7 specimens, Fig. 2A–I]. Two to four annular body swellings with coelomocytes in anterior half: first swelling at 3.3 mm; second swelling at 5.8 mm from anterior extremity in one male (body length, 15 mm) (Fig. 2C). Body length 18 (14–20) [17] mm; width at midbody 75 (75–90) [82]. Nerve-ring 245 (175–270) [227] from anterior extremity. Oesophagus 530 (470–670) [549] long. Apex of testis round, at 560 (450–1000) [590] from anterior extremity, situated near oesophago-intestinal junction (Fig. 2B); apex of testis anterior to oesophago-intestinal junction in three specimens, posterior to it in four specimens. Area rugosa precloacal, its transverse bands composed of one row of very small cuticular bosses; bands 1–2.5 apart; area rugosa extending from 95 to 238 from tail end in one male (body length, 15 mm); from 113 to 625 in another male (body length, 19 mm). Caudal alae narrow. Caudal papillae arranged in two ventrolateral groups and one precloacal ventral papilla: two precloacal pairs (pairs 1 and 2) and three postcloacal pairs (pairs 3–5) (Fig. 2F). One subventral asymmetrically arranged pair 6. On the posterior part of the tail, one ventrolateral pair 7 present (Fig. 2G). One small pair 8 present near lateral lappets (Fig. 2H). Right spicule 183 (132–183) [157] long, cylindrical in its proximal two thirds, becoming flattened and spatulate distally, without dorsal heel. Left spicule 490 (387–550) [467] long, simple with pointed tip, divided into blade and lamina. Left to right spicule ratio 2.2–3.7:1. Gubernaculum absent. Tail 98 (75–98) [85] long. Tail tip with one pair of subterminal conical lappets and axial point divided at apex (Fig. 2I).

3.1.3. Prevalence and intensity of filariae

Adults of *M.* (*Tup.*) *dunni* were found in 10 out of 45 (22.2%) common treeshrews collected from five research areas in Peninsular Malaysia. Microfilariae of *M.* (*Tup.*) *dunni* were present in the blood of 13 of these animals (28.9%). Specimens of *M.* (*Tup.*) *dunni* (79 adults: 66 females and 13 males) were obtained from the subcutaneous connective tissues of the neck and abdomen below the axillae. Intensity of infection ranged from 1 to 20 with a mean of 7 worms per infected host.

3.1.4. Remarks

We compared the morphological features of the present specimens with descriptions of *M.* (*Tup.*) *dunni* by previous authors (Mullin and Orihel, 1972; Eberhard and Orihel, 1984; Bain et al., 2015) and noticed some differences concerning the position of the vulva and features of the area rugosa. In the original description by Mullin and Orihel (1972), the vulva was stated to be located near the base of the oesophagus, but the metrics of the holotype were not recorded. However, the range and mean of the distance of the vulva from the anterior extremity were shorter than the length of the oesophagus. Subsequently, Eberhard and Orihel (1984) gave the position of the vulva of *M.* (*Tup.*) *dunni* as located at or posterior to the base of the oesophagus. In contrast, in the key to the subgenera of *Mansonella* presented by Bain et al. (2015), *Tupainema* is placed in the group of subgenera in which the vulva is located anterior to or in the region of the oesophago-intestinal junction. More specifically, the diagnosis for *Tupainema* gives the position of the vulva as at the level of or just posterior to the oesophago-intestinal junction (Bain et al., 2015). In the present study, the vulva was situated posterior to the junction in many of the longer females, but anterior to it in the shorter ones. We therefore suggest defining the subgenus *Tupainema* as

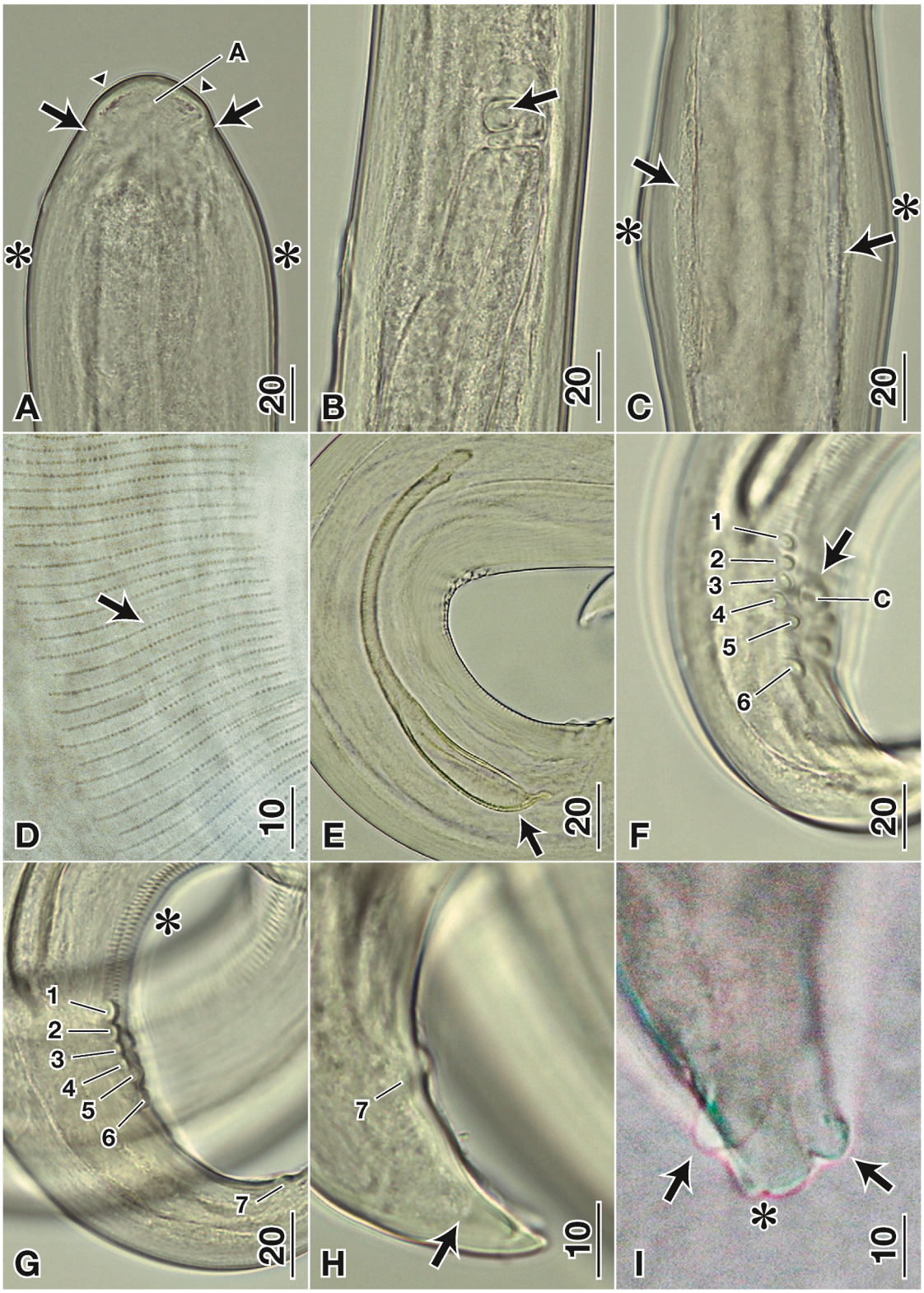

Fig. 2. Light micrographs of *Mansonella* (*Tupainema*) *dunni*. Males (**A-I**). **A** Anterior end slightly dilated, with conspicuous hemispherical prominence at apex, lateral view, showing amphid (*A*), labial papillae (*arrowheads*), cephalic papillae (*arrows*), and dilatation (*). **B** Apex of testis (*arrow*), lateral view. **C** Annular body swelling (*) with coelomocytes (*arrows*). **D** Area rugosa, oblique ventral view; transverse bands with a single row of small cuticular bosses (*arrow*). **E** Right spicule with spatulate distal part (*arrow*), lateral view. **F** Caudal papillae in two ventrolateral groups near cloacal aperture, oblique ventral view; precloacal ventral papilla (*arrow*), cloacal aperture (*C*) and 6 pairs of caudal papillae. **G** Posterior part of tail, lateral view, showing area rugosa (*), caudal papillae (6 pairs) and pair 7 in distal part. **H** Distal part of tail, lateral view, with caudal papilla (pair 7) and small papilla (*arrow*). **I** Tail tip, oblique ventral view, showing lateral conical lappets (*arrows*) and axial point broad with incision (*) at apex. Scale-bars are in micrometres.

possessing a vulva that is predominantly located posterior to the oesophago-intestinal junction.

In the subgenus *Cutifilaria*, which comprises the two species *M*. (*C*.) *perforata* and *M*. (*C*.) *wenki*, the vulva is situated markedly posterior to the oesophago-intestinal junction (Bain and Schulz-Key, 1974; Uni et al., 2004), and in the subgenus *Pseudolitomosa* at the junction (Yamaguti, 1941). In the remaining subgenera *Mansonella*, *Esslingeria* and *Tetrapetalonema* the vulva is situated at the mid-level of the oesophagus (Eberhard and Orihel, 1984; Bain et al., 2015).

In the present specimens, the area rugosa is composed of a single row of tiny cuticular bosses (Fig. 2D). Bain et al. (2015) described the area rugosa in *M*. (*Tup*.) *dunni* as composed of very short longitudinal cuticular crests and used these cuticular projections to differentiate subgenera in the key: pointed cuticular rugosities in *Cutifilaria vs* short longitudinal crests in *Mansonella*, *Esslingeria*, *Pseudolitomosa*, *Tetrapetalonema* and *Tupainema*; males of *Filyamagutia* are unknown. However, we found these projections to be very small and almost too subtle to examine in detail. Consequently, we suggest that the key to the subgenera of *Mansonella* be modified to reflect that the subgenus *Cutifilaria* possesses an area rugosa with transverse bands composed of irregularly disposed cuticular bosses, whereas the remaining subgenera, including the subgenus *Tupainema*, are characterized by an area rugosa with

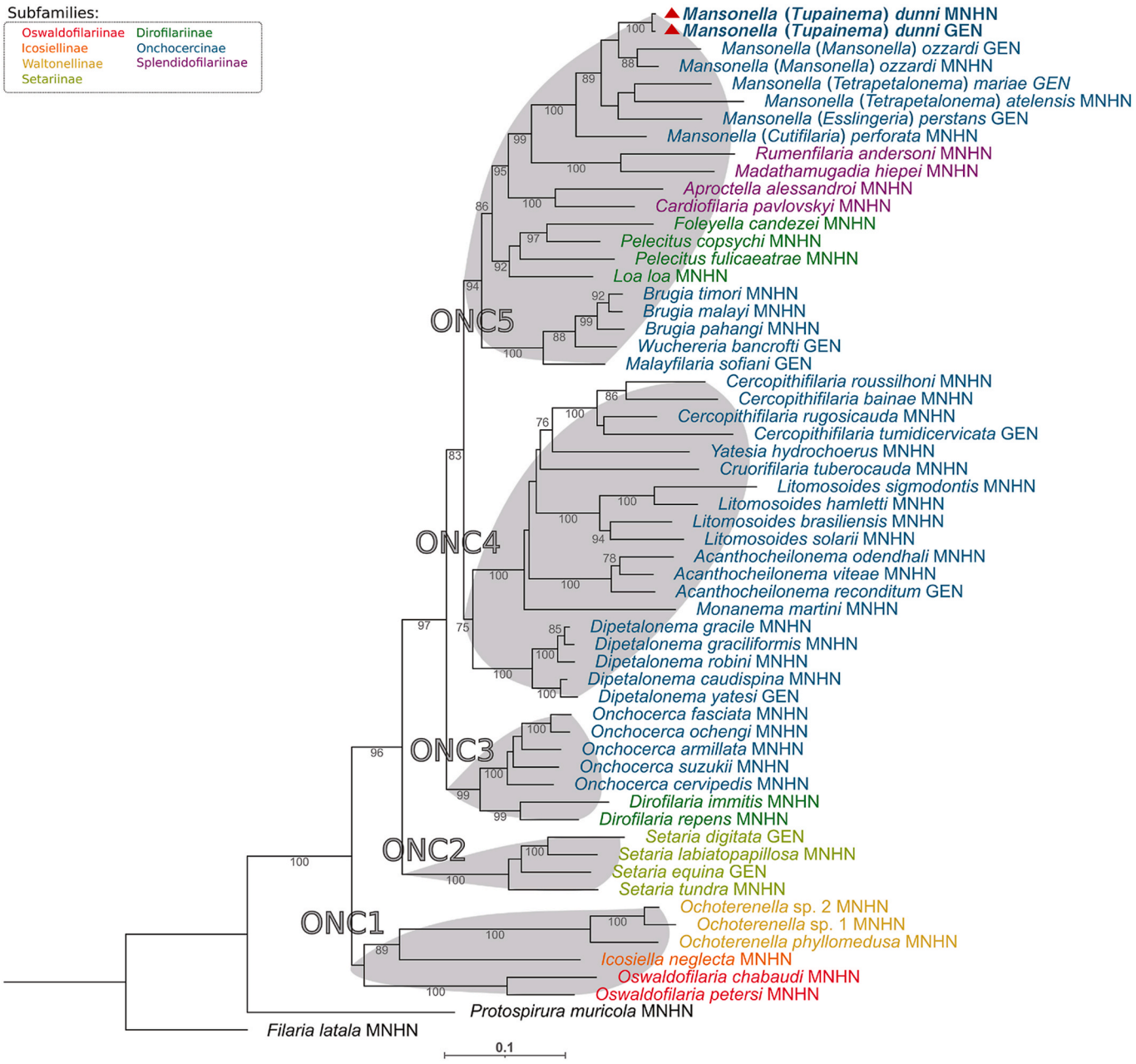

Fig. 3. Onchocercid clades based on partitioned concatenated datasets of *12S* rDNA, *cox*1, *rbp*1, *hsp70*, *myoHC*, *18S* rDNA and *28S* rDNA sequences using maximum-likelihood inference. The total length of datasets is approximately 3695 bp. Fifty-seven onchocercid sequences (representing 55 species) were analysed. *Filaria latala* and *Protospirura muricola* were used as the outgroup. The topology was inferred using 1000 bootstrap replications. The onchocercid subfamilies are indicated by colour: blue for Onchocercinae, dark green for Dirofilariinae, purple for Splendidofilariinae, pale green for Setariinae, yellow for Waltonellinae, orange for Icosiellinae and red for Oswaldofilariinae. The red triangles indicate the sequences generated in this study. The scale-bar below the diagram indicates the number of inferred changes along each branch. *Abbreviations*: MNHN, sequences were analysed at the Museum National d'Histoire Naturelle, Paris. GEN, sequence data were obtained from GenBank.

transverse bands composed of a single row of cuticular bosses or short longitudinal crests.

We recorded annular body swellings in both sexes of *M*. (*Tup*.) *dunni*. In contrast, such swellings are absent in the congeners *M*. (*C*.) *perforata*, *M*. (*C*.) *wenki*, *M*. (*P*.) *musasabi* (Yamaguti, 1941), *M*. (*Tet*.) *colombiensis* (Esslinger, 1982) and *M*. (*Tet*.) *panamensis* (McCoy, 1936) (Yamaguti, 1941; Bain and Schulz-Key, 1974; Uni et al., 2004; Bain et al., 2015).

Generally speaking, the area rugosa is a variable feature in males of the Onchocercinae. It has been recorded in species of *Mansonella* and *Cercopithifilaria*, but not *Onchocerca* (Bain et al., 2015; Uni et al., 2001, 2004, 2007, 2020). Bain and Chabaud (1988) suggested that body swellings and the area rugosa assist males in holding the female during copulation.

3.2. Filarial cox1 gene analysis

Calculated uncorrected p-distances for the *cox*1 sequences between two specimens (M1: KY434306 and G4: KY434309) of *M*. (*Tup*.) *dunni* collected from two different states (Johor and Selangor, respectively) were 0.42%. The genetic distances between *M*. (*Tup*.) *dunni* and its congeners were 13.09% for *M*. (*M*.) *ozzardi* (KP760195), 15.05% for *M*. (*Tet*.) *mariae* (KX932483), 15.6% for *M*. (*E*.) *perstans* (AM749265), 16.7% for *M*. (*Tet*.) *atelensis amazonae* (AM749278) and 15.6–16.15% for *M*. (*C*.) *perforata* (AM749265). The genetic distances between *Brugia malayi* (Brug, 1927) (MK250713) and *Wuchereria bancrofti* (Cobbold, 1877) (JQ316200) were 9.06%.

3.3. Molecular phylogeny of the Onchocercidae

Together with its congeners, *Mansonella* (*Tup*.) *dunni* formed a well-supported (100%) monophyletic group in clade ONC5 (Fig. 3). The latter was divided into five groups. The most basal group included *Malayfilaria sofiani* Uni et al., 2017, *W. bancrofti* and *Brugia* spp. (Onchocercinae); the second group comprised species of the Dirofilariinae, i.e., *Loa loa* (Cobbold, 1864), *Pelecitus* spp. and *Foleyella candezei* (Fraipont, 1882). Two further well-supported groupings (100%) contained two species of the Splendidofilariinae each: *Cardiofilaria pavlovskyi* Strom, 1937 and *Aproctella alessandroi* Bain et al., 1981; as well as *Madathamugadia hiepei* Hering-Hagenbeck et al.,2000 and *Rumenfilaria andersoni* Lankester & Snider, 1982. The most derived group within ONC5 was composed of species of *Mansonella*, with *M*. (*C*.) *perforata* at the basis of this clade and *M*. (*Tup*.) *dunni* in the most derived position, forming a sister clade to *M*. (*M*.) *ozzardi*. In the phylogenetic tree based on ITS1 nucleotide sequences (Supplementary Fig. S1), *M*. (*Tup*.) *dunni* was also closely related to *M*. (*M*.) *ozzardi*.

3.4. Wolbachia phylogeny

Mansonella (*Tup*.) *dunni* harboured endosymbionts of the *Wolbachia* supergroup F genotype (Fig. 4). This is in keeping with other congeners that have been screened for *Wolbachia* to date. The *Wolbachia* genotype of *M*. (*Tup*.) *dunni* was closely related to that of *M*. (*M*.) *ozzardi*, but distant from that of *M*. (*C*.) *perforata*. The supergroup F genotype includes endosymbionts of both filarial species and arthropods: the filariae being represented by species of *Mansonella* (Onchocercinae) and *M. hiepei* (Splendidofilariinae).

4. Discussion

According to Eberhard and Orihel (1984), the morphological features of *Mansonella* spp., including the absence of a pre-oesophageal cuticular ring, the weakly developed oesophagus, spicule morphology, absence of a gubernaculum, arrangement of pericloacal papillae and unsheathed microfilariae, suggest that *Mansonella* is a highly evolved genus within the Onchocercidae. Indeed, in the present molecular phylogeny, *Mansonella* spp. were positioned in the most derived group in clade ONC5 (Fig. 3). Comparing the six congeners in five subgenera that were available for our molecular analyses, we found certain morphological affinities between *M*. (*Tup*.) *dunni* and *M*. (*M*.) *ozzardi*. In addition to the generic characteristics mentioned above, the two species share the presence of annular body swellings, a female tail with two lateral lappets and divided axial points, and an area rugosa with transverse bands of very short cuticular crests in *M*. (*M*.) *ozzardi* and pointed bosses in *M*. (*Tup*.) *dunni* (Orihel and Eberhard, 1982; Eberhard and Orihel, 1984; Bain et al., 2015). The microfilariae of M. (*Tup*.) *dunni* and *M*. (*M*.) *ozzardi* are remarkably similar in that the tip of the tail is without a nucleus or nuclei. In contrast, species of the subgenera *Tetrapetalonema*, *Esslingeria* and *Cutifilaria* have microfilariae with nuclei

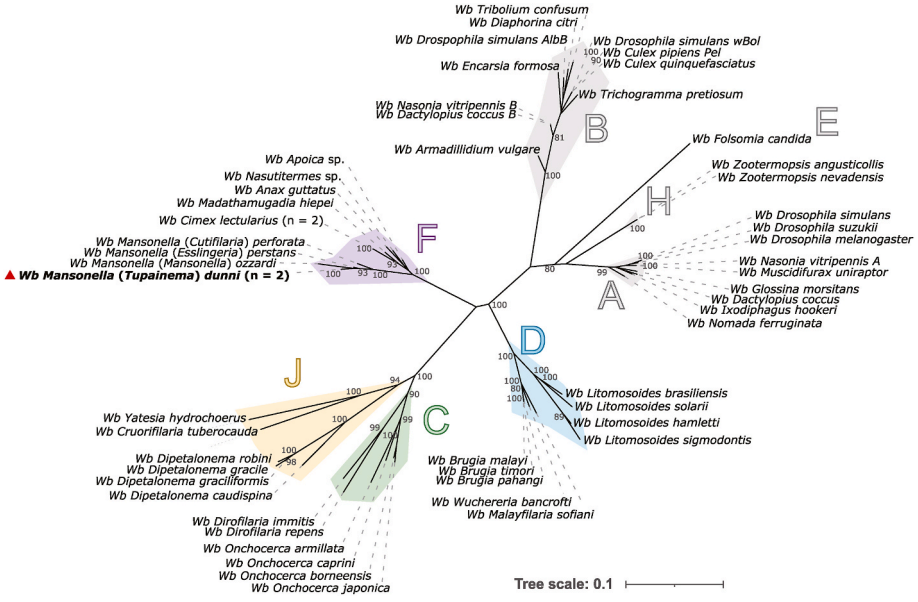

Fig. 4. Phylogenetic tree of *Wolbachia* endosymbionts based on six markers using maximum-likelihood inference. Analysis based on concatenation of *16S* rDNA, *ftsZ*, *dnaA*, *coxA*, *fbpA* and *gatB*. The dataset was partitioned to implement the best-fitting substitution model for each gene. The robustness of nodes was assessed with 1000 bootstrap replicates. The *Wolbachia* supergroups (A-F, H and J) were identified according to Lefoulon et al. (2016) and Lo et al. (2002). The red triangle indicates the sequence generated in this study. The scale-bar indicates the distance in substitutions per nucleotide. *Abbreviation*: *Wb*, *Wolbachia*.

extending to the tip of the tail (Eberhard and Orihel, 1984; Uni et al., 2004; Bain et al., 2015). The predilection sites for adults and microfilariae are also similar in both species (Orihel and Eberhard, 1982; Eberhard and Orihel, 1984). However, the arrangement of the papillae on the cephalic plate and the position of the vulva differs between the two species. In addition, the male tail of *M*. (*M*.) *ozzardi* bears a cuticular flap; that of *M*. (*Tup*.) *dunni* has two subterminal lappets.

Mansonella (*C*.) *perforata* on the other hand, differs from *M*. (*Tup*.) *dunni* in the following features: markedly posterior position of the vulva, absence of body swellings, male tail without lateral lappets, structure of the right spicule, transverse bands of the area rugosa consisting of irregularly arranged pointed rugosities and presence of nuclei at the bifid tail end of microfilariae (Uni et al., 2004; Bain et al., 2015). In addition, the predilection site (dermis) for both adults and microfilariae of *M*. (*C*.) *perforata* differs from that of *M*. (*Tup*.) *dunni* (subcutaneous tissues for adults and blood for microfilariae) (Uni et al., 2004). The morphological distinctness of *M*. (*C*.) *perforata* is corroborated by its basal position in the phylogenetic tree (Fig. 3), when compared to its congeners.

The presence or absence of a pre-oesophageal cuticular ring and the division of the oesophagus appear to be morphological features that are generally related to the molecular phylogeny of ONC5 (Fig. 3). In the species of *Mansonella*, a pre-oesophageal ring is absent and the oesophagus is poorly developed and undivided. In the next group, both *M. hiepei* and *R. andersoni* have no pre-oesophageal ring, the oesophagus is clearly divided in the former and indistinctly divided in the latter species (Lankester and Snider, 1982; Hering-Hagenbeck et al., 2000). In *C. pavlovskyi*, the pre-oesophageal ring is present, and the oesophagus is undivided (Bartlett and Anderson, 1980); in its sister taxon *A. alessandroi*, the pre-oesophageal ring is also present, but the oesophagus is divided (see figure 1 of Bain et al., 1981). In the group of *Foleyella* and *Pelecitus*, the presence or absence of the pre-oesophageal ring and the division of the oesophagus varies with the species (Bartlett, 1986; Bartlett and Greiner, 1986). Finally, in *Brugia* spp., *Wuchereria* spp. and *M. sofiani*, both the pre-oesophageal ring and division of the oesophagus are present (Uni et al., 2017).

The genetic distance between sequences of the *cox*1 gene of *M*. (*Tup*.) *dunni* and other congeners was 13.09% for *M*. (*M*.) *ozzardi* and 15.6–16.15% for *M*. (*C*.) *perforata*. According to Ferri et al. (2009), filariae can be considered different species if the genetic distance based on the *cox*1 sequences is greater than 4.8%. In *Onchocerca* spp., *cox*1 interspecific distances are higher than 4.5% and intraspecific distances are lower than 2% (Lefoulon et al., 2017). Therefore, we consider the distances (0.42%) between the two specimens (M1 from Johor and G4 from Selangor) of *M*. (*Tup*.) *dunni* collected from different states of Malaysia intraspecific. It is noteworthy that the interspecific distances between *Mansonella* spp. were rather large in comparison with the generic distance (9.06%) between *B. malayi* and *W. bancrofti*, both placed in the first group of clade ONC5. *Mansonella* is a large genus with 29 species, while *Brugia* and *Wuchereria* are small genera with 11 and two species, respectively (Bain et al., 2014). *Brugia* spp. have close affinities to *W. bancrofti*, based on the morphological characteristics of both adults and infective larvae, their development, and transmission (Anderson, 2000). In the molecular phylogenetic analyses of filariae and their *Wolbachia* endosymbionts, *Brugia* species are also closely associated with *W. bancrofti* (Casiraghi et al., 2001). Ramesh et al. (2012) estimated that *Brugia* and *Wuchereria* have diverged some 675,000 years ago; a relatively recent split in the superfamily Filarioidea according to McNulty et al. (2013). We speculate that *Mansonella* has a complex evolutionary history and attribute its large genetic divergence to its worldwide geographical distribution and broad host spectrum (11 families in five orders of Mammalia).

Mansonella spp. available for the present molecular analysis (Fig. 3) have a diverse host and geographical range. The host of *M*. (*Tup*.) *dunni* is the common treeshrew in Malaysia, while its closest relative, *M*. (*M*.) *ozzardi*, infects humans in Central and South America as well as the Caribbean Islands (Bain et al., 2015). Platyrrhine monkeys are hosts of *M*. (*Tet*.) *mariae* and *M*. (*Tet*.) *atelensis amazonae* in Guyana and Venezuela, respectively (Petit et al., 1985; Bain et al., 2015), while *M*. (*E*.) *perstans* infects humans in Africa and South America. *Mansonella* (*C*.) *perforata*, on the other hand, parasitizes sika deer in Japan (Uni et al., 2004; Nagata, 2009). As for other congeners, *M*. (*Pseudolitomosa*) *musasabi* (Yamaguti, 1941) was described from the Japanese giant flying squirrel *Petaurista leucogenys* (Temminck) (Rodentia: Sciuridae) and *M*. (*Filyamagutia*) *akitensis* (Uni, 1983) from the Japanese black bear *Ursus thibetanus* G. Cuvier (Carnivora: Ursidae) (Yamaguti, 1941; Uni, 1983). In South America, two species of *Esslingeria*, *M*. (*E*.) *rotundicapita* Eberhard et al., 1984 and *M*. (*E*.) *longicapita* Eberhard et al., 1984, were described from the Venezuelan capybara *Hydrochoerus hydrochaeris* L. (Rodentia: Caviidae) (Eberhard et al., 1984), but the remainder of species within this subgenus are parasites of humans and African anthropoids (Bain et al., 1995, 2015). Contrary to this, all species of the subgenus *Tetrapetalonema* are found in platyrrhine monkeys in South America (Bain et al., 1986, 2015). Within *Esslingeria*, both adults and microfilariae of *M*. (*E*.) *rotundicapita* and *M*. (*E*.) *longicapita* from capybaras in South America had similar morphological features and predilection sites (the dermis) as adults and microfilariae of *M*. (*E*.) *streptocerca* and *M*. (*E*.) *rodhaini* in humans and anthropoid apes in Africa (Eberhard et al., 1984).

Recently, Poux et al. (2006) suggested a scenario for the arrival of primates and caviomorphs in South America by a trans-Atlantic migration from Africa at the end of the Eocene (< 45 Mya), followed by the radiation of extant platyrrhines during the Early Miocene (> 16 Mya). According to Bain (2002), *M*. (*E*.) *perstans* was introduced into South America by human migration from Africa. In contrast, *M*. (*M*.) *ozzardi* originated from host switching of parasites of carnivores or sciurids in North America. The most ancestral form of the *Mansonella* lineage likely existed in the Asiatic region and its hosts migrated towards Africa through the Arabian Peninsula. On the other hand, some of the hosts in Asia migrated towards North America *via* the Bering Strait (Bain, 2002). In North America, *M*. (*M*.) *llewellyni* (Price, 1962) was described from the raccoon *Procyon lotor* (L.) (Carnivora: Procyonidae) and *M*. (*M*.) *interstitium* (Price, 1962) from the gray squirrel *Sciurus carolinensis* Gmelin (Rodentia: Sciuridae) (Price, 1962). In Asia, *M*. (*P*.) *musasabi*, *M*. (*F*.) *akitensis* and *M*. (*C*.) *perforata* were found in the giant flying squirrel, the black bear and the sika deer, respectively, emphasizing the heterogenous host spectrum and wide geographical distribution of *Mansonella* spp.

Considering the origin of treeshrews, the common ancestor diverged into Scandentia, Dermoptera and Primates during the Cretaceous (90 Mya), and the genus *Tupaia* Raffles, 1821 arose at the end of the Miocene (10 Mya) (Janečka et al., 2007). Phylogenetically, treeshrews (Scandentia) are considered more closely related to Primates than to Rodentia and Lagomorpha (Springer et al., 2004). Roberts et al. (2011) suggested that Miocene tectonic events, volcanism and geographical instability drove treeshrew diversification in Southeast Asia. Therefore, we speculate that treeshrews acquired an ancestral form of *Mansonella* through host switching of *Mansonella* forms in sciurids, carnivores or ruminants distributed in the Holarctic Region. Moreover, host specificity was not as strong during the Pleistocene (< 2.58 Mya) as originally claimed (Krueger et al., 2007). We further posit that host switching was facilitated by haematophagous arthropods serving as vectors for these filariae.

To date, no investigation concerning possible vectors of *M*. (*Tup*.) *dunni* has been carried out, although 62 species of *Simulium* and 108 species of *Culicoides* have been reported from Peninsular Malaysia (Wirth and Hubert, 1989; Takaoka et al., 2018). Tidwell et al. (1980) established the *S. sanguineum* group as one of the main vectors of *M*. (*M*.) *ozzardi* in the Mitú region in Columbia. Orihel et al. (1981) harvested infective larvae of *M*. (*M*.) *ozzardi* from *C. furens* (Poey) collected in Haiti as well as from *Simulium* sp. (*sanguineum* group) collected in the Colombian Amazon and experimentally obtained adult worms from

patas monkeys *Erythrocebus patas* (Schreber) (Primates: Cercopithecidae). In North America, Yates et al. (1982) obtained larval stages of *M.* (*M.*) *llewellyni* in the thoracic muscles of *C. hollensis* (Melander & Brues) fed on the blood of a raccoon infected with the filarial parasites.

Interestingly, *M.* (*Tup.*) *dunni* from common treeshrews held the most derived position among its congeners in our molecular analyses. Ultimately, finding the ancestral forms of the *Mansonella* lineage will necessitate molecular analysis of other related filariae such as *S. sunci* from the Asian musk shrew *Suncus murinus* (L.) (Soricomorpha) in the Indomalayan realm (Hutterer, 2005; Morales-Hojas, 2009; Bain et al., 2015).

Regarding the phylogenetic relationships between *Wolbachia* supergroups and host species of the Onchocercidae, an ancestral absence of *Wolbachia*, horizontal acquisitions, secondary losses, and local coevolution with host filariae are all scenarios that have been suggested to date (Bain et al., 2008; Ferri et al., 2011; Lefoulon et al., 2012, 2016; Uni et al., 2020). In this study, *M.* (*Tup.*) *dunni* harboured *Wolbachia* of the supergroup F genotype. Lefoulon et al. (2016) suggested an absence of global coevolution between filarial worms and their *Wolbachia* endosymbionts; while strong coevolution is found in the relationships between the supergroups C and J and their filarial hosts, weak coevolution is seen in the relationships between the supergroups D and F and their filarial hosts. In our study, the *Wolbachia* genotype of *M.* (*Tup.*) *dunni* was closely related to that of *M.* (*M.*) *ozzardi*, but distant from that of *M.* (*C.*) *perforata* (Fig. 4), reflecting the phylogenetic relationships of these congeners.

5. Conclusions

We redescribed the morphological features of *M.* (*Tup.*) *dunni* obtained from common treeshrews in Malaysia. Based on this, we suggested modifications to the key for the subgenera of *Mansonella* proposed by Bain et al. (2015) concerning the position of the vulva and the composition of the transverse bands of the area rugosa. Morphological analysis revealed that *M.* (*Tup.*) *dunni* shares certain morphological features with *M.* (*M.*) *ozzardi* but differs distinctly from *M.* (*C.*) *perforata*. Molecular analyses indicated that species of *Mansonella* constitute a monophyletic group in clade ONC5 of the Onchocercidae, and *M.* (*Tup.*) *dunni* is one of the most recently derived filariae. *Mansonella* (*Tup.*) *dunni* formed a sister clade to *M.* (*M.*) *ozzardi* from humans and was most distant from *M.* (*C.*) *perforata* from sika deer in the newly generated phylogenetic tree. Hence, we consider that the molecular phylogeny of *Mansonella* species corroborates their morphological differences. *Wolbachia* endosymbionts of the supergroup F genotype were detected in *M.* (*Tup.*) *dunni*. The *Wolbachia* genotype of *M.* (*Tup.*) *dunni* was closely related to that of *M.* (*M.*) *ozzardi*, but distant from that of *M.* (*C.*) *perforata*.

Funding

This study was supported by the Ministry of Higher Education, Malaysia (FRGS FP020-2012A).

Ethical approval

Culling of animals and all experimental procedures were carried out in strict compliance with the policy and protocols approved by the Institutional Animal Care and Use Committee, Universiti Malaya, Kuala Lumpur, Malaysia (Protocol No. S/15102018/31082018-02/R). The surveys were carried out in accordance with the conservation and control policies of the Department of Wildlife and National Park Malaysia, Malaysia (Permission No. W-00660-16-16).

CRediT authorship contribution statement

Ahmad Syihan Mat Udin: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Shigehiko Uni:** Supervision, Writing – review & editing, Visualization, Funding acquisition. **Jules Rodrigues:** Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Coralie Martin:** Data curation, Resources, Validation, Writing – review & editing. **Kerstin Junker:** Conceptualization, Writing – review & editing, Validation. **Takeshi Agatsuma:** Methodology, Formal analysis, Visualization, Writing – review & editing. **Van Lun Low:** Methodology, Formal analysis, Writing – review & editing. **Weerachai Saijuntha:** Methodology, Validation, Writing – review & editing. **Hasmahzaiti Omar:** Investigation, Methodology, Writing – review & editing. **Nur Afiqah Zainuri:** Investigation, Resources, Writing – review & editing. **Masako Fukuda:** Methodology, Formal analysis, Writing – review & editing. **Makoto Matsubayashi:** Methodology, Resources, Writing – review & editing. **Daisuke Kimura:** Methodology, Resources, Writing – review & editing, Validation. **Hiroyuki Takaoka:** Investigation, Validation, Writing – review & editing. **Rosli Ramli:** Supervision, Resources, Writing – review & editing, Validation. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All newly generated gene sequences of *M.* (*Tup.*) *dunni* (F1) were deposited in the GenBank database under the following accession numbers: OR198850 (*cox*1), OR210206 (*12S* rDNA), OR198492 (*18S* rDNA), OR555759 (*MyoHC*), OR555767 (*hsp70*), OR555760 (*rbp*1) and OR206512 (*28S* rDNA). Additional sequence data for specimens (M1-M2; G3-G4) of *M.* (*Tup.*) *dunni*: M1: KY434306 (*cox*1), KY434310 (*12S* rDNA); M2: KY434307 (*cox*1), KY434311 (*12S* rDNA); M2 C1: KY434312 (ITS1); M2 C2: KY434313 (ITS1); M2 C3: KY434314 (ITS1); G3: KY434308 (*cox*1); and G4: KY434309 (*cox*1). Sequence data for the *Wolbachia* endosymbionts of *M.* (*Tup.*) *dunni*: M1: OR555761 (*dnaA*), OR555763 (*ftsZ*) and OR555765 (*gatB*); M2: OR555762 (*dnaA*), OR555764 (*ftsZ*) and OR555766 (*gatB*). Voucher specimens are deposited in the Museum National d'Histoire Naturelle (MNHN), Paris, France, under accession numbers MNHN-IN-110YT for the female of *M.* (*Tup.*) *dunni* and MNHN-IN-111YT for the male. Other specimens are deposited in the Museum of Zoology, Institute of Biological Sciences, Universiti Malaya, under accession numbers MdF-1–7 and MdM-1–3.

Acknowledgements

We are grateful to Academician Dr Yong Hoi Sen, Senior Fellow of the Academy of Science Malaysia and Professor Emeritus of the Universiti Malaya, who warmly encouraged us in our research efforts. We thank Drs Mohd Sofian Azirun and Rosli Hashim, Professors of the Universiti Malaya, who conducted the Scientific Inventory in Ulu Gombak Field Studies Centre, Universiti Malaya, Selangor.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2023.100154.

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