Current Research in Parasitology & Vector-Borne Diseases 2 (2022) 100078

Contents lists available at ScienceDirect

Current Research in Parasitology & Vector-Borne Diseases

journal homepage: www.editorialmanager.com/crpvbd/default.aspx

Description and molecular characterisation of *Pelecitus copsychi* Uni, Mat Udin & Martin n. sp. (Nematoda: Onchocercidae) from the white-rumped shama *Copsychus malabaricus* (Scopoli) (Passeriformes: Muscicapidae) of Pahang, Malaysia

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ARTICLE INFO

Keywords:
Dirofilariinae
Foleyella candezei
Multi-locus sequence analyses
Indomalaya
Pelecitus fulicaeatrae

ABSTRACT

Species of the genus *Pelecitus* Railliet & Henry, 1910 the most widely distributed avian filariae in Africa and South America. Zoonotic cases in humans were reported in South America. While investigating the filarial fauna of wild animals in Malaysia, we discovered an undescribed filaria from the swollen footpad of the left leg of *Copsychus malabaricus* (Scopoli) in Pahang, Peninsular Malaysia. Adults of both sexes have a corkscrew-shaped body. Based on comparison of their morphological characteristics (i.e. pre-oesophageal cuticular ring distinct, oesophagus divided, vulva protuberant and situated at the level of anterior half of oesophagus, spicules strongly sclerotized and left spicule with broad blade) with other *Pelecitus* species, they are here described as *Pelecitus copsychi* Uni, Mat Udin & Martin n. sp. Multi-locus sequence analyses based on seven genes (*12S* rDNA, *cox*1, *18S* rDNA, *28S* rDNA, *MyoHC*, *rbp1* and *hsp70*) were performed to determine the phylogenetic position of the new species. The calculated p-distance between the *cox*1 gene sequences for *P. copsychi* n. sp. and *Pelecitus fulicaeatrae* (Diesing, 1861) was 14.1%. Intraspecific genetic variation between two individuals of the new species was 0.4%. In both the Bayesian inference and maximum-likelihood trees, *P. copsychi* n. sp. was positioned in the second clade of ONC5, containing three genera of the subfamily Dirofilariinae (*Foleyella* Seurat, 1917, *Pelecitus* and *Loa* Stiles, 1905). Immunostaining and molecular analyses remained negative for the presence of *Wolbachia* endosymbionts. Our findings corroborate the division of the subfamily Dirofilariinae into ONC3 with *Dirofilaria* Railliet & Henry, 1911 and ONC5 with *Pelecitus*.

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https://doi.org/10.1016/j.crpvbd.2022.100078
Received 30 December 2021; Received in revised form 21 January 2022; Accepted 24 January 2022

1. Introduction

Filarial nematodes within the family Onchocercidae Leiper, 1911 that infect avian hosts are an important taxonomic group. Indeed, based on morpho-anatomical criteria, more than one third of the approximately 600 described filarial species are avian filariae, divided into 16 genera (Anderson & Bain, 1976; Bain, 2002; Bartlett, 2008). Among them, only the genus *Pelecitus* Railliet & Henry, 1910 belongs to the subfamily Dirofilariinae Sandground, 1921, which includes the human filaria *Loa loa* (Cobbold, 1864) and the canine filaria *Dirofilaria immitis* (Leidy, 1856) (Anderson & Bain, 1976). The host spectrum of all avian filarial genera is limited to birds, except for *Pelecitus*, which includes three species that parasitize mammals (Bartlett & Greiner, 1986; Bartlett, 2008). The genus *Pelecitus* is cosmopolitan, with currently 20 species considered valid. Most of these are distributed in Africa and South America, except for *Pelecitus fulicaeatrae* (Diesing, 1861), which has been reported worldwide (Vanderburgh et al., 1984; Bartlett & Greiner, 1986) (see Supplementary Table S1 for details).

Bartlett & Greiner (1986) revised the genus *Pelecitus*. They retained 14 species as valid and also transferred two filarial species parasitic in mammals that had previously been placed in the genus *Dirofilaria* Railliet & Henry, 1911 to *Pelecitus*. Since then, a further two species, *Pelecitus spiralis* (Annett, Dutton & Elliott, 1901) from *Ploceus aurantius* (Vieillot) (Passeriformes) and *Pelecitus major* (Annett, Dutton & Elliott, 1901) from *Ploceus nigricollis brachypterus* Swainson in Nigeria, were assigned to *Pelecitus* (see Bartlett & Anderson, 1987a). Furthermore, two new congeners were described. In Mexico, *Pelecitus meridionaleporinus* Jiménez-Ruiz, Gardner, Cervantes & Lorenzo, 2004 was described from *Lepus flavigularis* Wagner (Lagomorpha) (Jiménez-Ruiz et al., 2004), and in Australia, *Pelecitus bartneri* Spratt, 2010 was described from *Psephotus chrysopterygius* Gould (Psittaciformes) (Spratt, 2010; Supplementary Table S1).

The pathology caused by *Pelecitus* spp. in their avian hosts includes swollen joints, lameness and chronic inflammatory tenosynovitis (Greve et al., 1982; Allen et al., 1985; Bartlett, 2008). Adult worms are generally localized in the tendons of the legs and feet, sometimes in the muscles of the legs or in the articulations of the tibia or knee joint (Bartlett & Greiner, 1986; Supplementary Table S1). Recently, Muñoz-García et al. (2018) reported a severe tenosynovitis caused by *Pelecitus* sp. found in swollen nodules on both legs of a crested caracara *Caracara cheriway* (von Jacquin) with signs of pain in Mexico. Rare cases of zoonotic infections caused by *Pelicitus* sp. have been described in humans (Bortero et al., 1984; Bain et al., 2011). Bain et al. (2011) reported *Pelecitus* sp. from the iris of a man in Brazil and suggested that specimens that had been recovered from the anterior chamber of the eye of a patient in Colombia and identified as *Loaina* sp. by Bortero et al. (1984) belonged to *Pelecitus* as well.

In Indomalaya, *Pelecitus ceylonensis* Dissanaike, 1967 was recovered from two naturally infected crows (Passeriformes) as well as from experimentally infected ash doves (Columbiformes) and chickens (Galliformes) in Sri Lanka (Dissanaike, 1967; Supplementary Table S1). In addition, *Pelecitus galli* Dissanaike & Fernando, 1974 was described from *Gallus gallus spadiceus* (Bonnaterre) (Galliformes) in Tasek Bera, Pahang, Malaysia (Dissanaike & Fernando, 1974). Although 36 species of filarial parasites from 22 genera have previously been recorded in vertebrates in Malaysia (Yen, 1983; Gibbons, 2010; Uni et al., 2017, 2020), *P. galli* is the only species of *Pelecitus* reported in Malaysia to date.

During a scientific inventory of the Krau Wildlife Reserve, Pahang, Malaysia, organized by the Department of Wildlife and National Parks, Malaysia in 2013, we collected nematodes from the swollen footpad of the leg of a white-rumped shama *Copsychus malabaricus* (Scopoli) (Passeriformes) caught in the primary forest. Herein, we describe the specimens as *Pelecitus copsychi* Uni, Mat Udin & Martin n. sp. and provide their molecular characterisation, revealing a close association with *P. fulicaeatrae* from birds and *Foleyella candezei* (Fraipont, 1882) from squamates in Africa (Bartlett, 1986).

A recent study on the phylogenetic relationships within the family Onchocercidae included few species of avian filariae, namely *Aproctella alessandroi* Bain, Petit, Kozek & Chabaud, 1981, *Cardiofilaria pavlovskyi* Strom, 1937 and *P. fulicaeatrae* (see Lefoulon et al., 2015). In the present study, newly generated as well as previously published partial sequences of two mitochondrial (*12S* rDNA and *cox*1) and five nuclear genes (*18S* rDNA, *28S* rDNA, the myosin heavy chain (*MyoHC*), RNA polymerase II large subunit (*rbp1*) and 70 kDa heat-shock proteins (*hsp70*)) were used to study the phylogenetic interrelationships within the Onchocercidae, including a wider range of avian filariae.

2. Materials and methods

2.1. Collection of hosts and parasites

A scientific inventory, organized by the Institute of Biodiversity, Department of Wildlife and National Parks, Malaysia, in Sungai Teris (3°36′42.6″N, 102°06′55.9″E), Krau Wildlife Reserve, Pahang, Malaysia, was undertaken between 21 May 2013 and 27 May 2013. During this inventory, a white-rumped shama (ID no. A8) with a swollen footpad of the left leg was examined for parasites. The swollen footpad was dissected under a stereomicroscope and 16 filarial parasites (12 females and 4 males) removed for subsequent morphological and molecular analyses.

2.2. Morphological study

Adult worms (8 females and 4 males) were fixed in 70% ethanol for morphological examination. Specimens were cleared in lactophenol (R & M Chemicals, Essex, UK) and drawn under a compound microscope equipped with a camera lucida (Olympus U-DA, Olympus, Tokyo, Japan). Measurements are in micrometres unless otherwise indicated and are given as the range; body length was measured following the helical coils. Thick blood smears were made and stained with 3% Giemsa solution (pH 7.4). One female fixed in 70% ethanol was embedded in paraffin, and sections were stained with haematoxylin and eosin (HE).

2.3. Molecular analysis of filarial nematodes

Four females were transferred directly into 80% ethanol and stored for molecular analysis. DNA was extracted from two female fragments (ID nos. 168-4 and 268-5) 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), *rbp1* (*c.*640 bp) and *hsp70* (*c.*610 bp). PCR reactions were processed in a final volume of 20 μl under the conditions summarised in Supplementary Table S2 (Casiraghi et al., 2001, 2004; Lefoulon et al., 2015).

Samples were sequenced at the Muséum National d'Histoire Naturelle (MNHN), using a MiSeq Illumina. Libraries were prepared following the protocol of Meyer & Kircher (2010). The reads were paired-ends with a theoretical size of 250 bp. For each gene, reads were mapped against the *P. fulicaeatrae* sequence (Supplementary Table S2) using Bowtie2 v2.4.1 (Langmead & Salzberg, 2012) with the following parameters: seed length of 18 bp; "very sensitive local" mode; mixed or discordant pair-end reads not allowed. The mapped reads were excluded if their length was inferior to 75 bp, or if more than 1% of their length was soft-clipped or if their edit distance was superior to 5% of their length. The remaining reads were assembled against their sequence of reference using IGV v2.9.4 (Robinson et al., 2011).

2.4. Filarial cox1 gene analysis

A DNA barcoding approach based on the *cox*1 marker was used to identify the specimens of *Pelecitus* from *C. malabaricus*. The *cox*1 marker was chosen over the *12S* rDNA marker for its greater robustness (fewer

indels, less susceptible to dataset variation) (Ferri et al., 2009). The *cox*1 sequence divergence was estimated by the number of base differences per site between two sequences (p-distance) using MEGA version 11 (Tamura et al., 2021). *Cox*1 sequences generated during the present study and those from the literature were analysed. Pairwise comparisons between the selected 48 *cox*1 sequences were processed, with each sequence representing a species (Supplementary Table S3).

2.5. Phylogenetic analyses

Sequences generated during the present study and those from the literature (Supplementary Table S3) were aligned using SATe v2.2.7 (Liu et al., 2009). Fifty-one sequences for 50 species belonging to 27 genera were included in the analyses and their phylogenetic relationships inferred using Bayesian inference as implemented in MrBayes 3 (Ronquist & Huelsenbeck, 2003). A partitioned model was implemented to estimate evolution parameters separately for each gene. For the analyses, the weighted average of the best-fit evolution models in the GTR+G landscape was used for each gene. Two runs were performed using five million steps with four chains, with tree sampling every 1000 generations; the first 25% of the trees were discarded as "burn-in" and posterior probabilities were calculated from the remaining trees.

In addition, a phylogeny based on the maximum-likelihood (ML) was implemented. 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 & Gascuel, 2003). The TPM1uf+I+G was the best fit for *12S* rDNA; TPM1+I+G for *18S* rDNA; TVM+I+G for *28S* rDNA; GTR+I+G for c*ox*1; TPM2uf+I+G for *hsp70*; TrN+G for *MyoHC* and TPM3uf+G for *rbp1*. To root the trees, two species were included as the outgroup: *Filaria latala* Chabaud & Mohammad, 1989 (Spirurida: Filariidae) and *Protospirura muricola* Gedoelst, 1916 (Spirurida: Spiruridae). The phylogenetic relationships of the Onchocercidae were inferred by the ML on the partitioned concatenated dataset. The program was executed by generating 10 random start trees with 1000 bootstraps using RaxML-NG (Kozlov et al., 2019).

2.6. Wolbachia detection

Sections of a female of *P. copsychi* n. sp. were stained with a rabbit polyclonal antiserum raised against the surface protein of *Wolbachia* from *Brugia pahangi* (Buckley & Edeson, 1956) (1:2000 dilution) as described by Kramer et al. (2003) and Ferri et al. (2011).

In addition, we screened for *Wolbachia* endosymbionts by nested PCR covering six genes (*16S* rDNA, *ftsZ*, *dnaA*, *coxA*, *fbpA* and *gatB*), as described by Lefoulon et al. (2016).

3. Results

3.1. *Pelecitus copsychi* Uni, Mat Udin & Martin n. sp.

3.1.1. Taxonomic summary

Type-host: *Copsychus malabaricus* (Scopoli) (Passeriformes: Muscicapidae), white-rumped shama.

Type-locality: Sungai Teris (3°36′42.6″N, 102°06′55.9″E), Krau Wildlife Reserve, Pahang, Malaysia.

Type-material: The holotype female (MNHN-IN-107YT) and allotype male (MNHN-IN-108YT) were deposited in the Muséum National d'Histoire Naturelle, Paris, France. Paratypes (7 females: Pf8-1–7; 3 males: Pm8-9–11) were deposited in the Institute of Biological Sciences, Universiti Malaya, Malaysia. Collection date: 24 May 2013.

Site in host: Adult worms occurred free in the swollen footpad of the left leg.

Representative DNA sequences: Sequence data for *P. copsychi* n. sp. were deposited in the GenBank database as follows: *cox*1 (OK480041 and OK480043), *12S* rRNA (OK480976 and OK480977), *18S* rRNA (OK481072 and OK481073), *MyoHC* (OK493401 and OK493402), *hsp70* (OK493398 and OK493399), *rbp1* (OK493403 and OK493404) and *28S* rRNA (OK481079 and OK481080).

ZooBank registration: To comply with the regulations set out in Article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:17738217-2468-49CF-A6CA-2208134E5209. The LSID for the new name *Pelecitus copsychi* Uni, Mat Udin & Martin n. sp. is urn:lsid:zoobank.org:act:8BF6174D-DE38-4F83-9F64-6768E8F8287C.

Etymology: The specific epithet is derived from the genus name of the type-host.

3.1.2. Description

General. [Table 1; Figs. 1 and 2.] Body corkscrew-shaped in both sexes, in form of dextral helix with 3–4 rotations (Fig. 1A). Body width uniform over most of length but tapering gradually at both ends. Cephalic papillae arranged in four submedian pairs, not markedly protuberant. Amphids small, lateral, not salient. Pre-oesophageal cuticular ring present and distinct (Figs. 1B and 2B). Oesophagus clearly divided into anterior muscular and posterior glandular portions (Fig. 2A, G). Both sexes with rounded posterior extremity. Lateral alae present, broadening towards posterior extremity in both sexes (Fig. 1D–F). Cuticle thick, with conspicuous, closely-spaced, transverse striations.

Female. [Based on 8 gravid specimens.] Vulva located at level of anterior half of oesophagus, roughly in region of junction between muscular and glandular oesophagus; ratio of distance of vulva from anterior extremity to total oesophagus length: 0.31–0.43. Vulva situated on protuberant cone of tissue (Figs. 1C and 2C). Vagina directed posteriorly. Post-deirid at 533–620 from caudal extremity, on left side (Figs. 1E and 2D).

Microfilaria. [Based on 10 specimens from anterior parts of uteri.] Loose sheath present, extending past anterior extremity of body. Extremities bluntly rounded (Fig. 2E and F). Microfilariae not found in thick blood smears of infected host.

Male. [Based on 4 specimens.] Caudal alae present (Figs. 1F and 2I, L); hyaline or granular inclusions not observed within caudal alae. Spicules well-sclerotized, unequal, dissimilar; right spicule short and slender, left spicule short and broad with well-defined handle and lamina (Figs. 1F and 2J, K). Caudal papillae arranged in two groups: 3 pairs of large pedunculate papillae, first two pairs precloacal, third pair adcloacal; and 3 pairs of small sessile postcloacal papillae. A single ventral precloacal papilla present (Fig. 2L). Post-deirids not observed.

3.1.3. Remarks

The present specimens were assigned to the genus *Pelecitus* (Onchocercidae: Dirofilariinae) as defined by Anderson & Bain (1976), Bartlett & Greiner (1986), Bartlett & Anderson (1987b) and Gibbons (2010), because of their corkscrew-shaped body, the presence of a pre-oesophageal cuticular ring and lateral alae that extend from the cervical region to the caudal extremity. Although not observed in males, a single post-deirid was found in females. Microfilariae are bluntly rounded at both extremities and possess a loose sheath. As is typical for the genus, the present specimens were associated with tendons and muscles near the leg joints of their host. To date, 17 species of *Pelecitus* have been recorded from birds (Bartlett & Greiner, 1986; Bartlett & Anderson, 1987a; Spratt, 2010; Supplementary Table S1).

We compared the morphological characteristics and morphometrics of the present specimens with three congeners reported in Indomalaya and Australia: *P. ceylonensis* in Sri Lanka, *P. galli* in Malaysia, and *P. bartneri* in Australia (Table 1). *Pelecitus copsychi* n. sp. is distinct from *P. galli* and *P. bartneri* in that its females are shorter, only reaching half the length of the latter two species. In contrast, both males and females of *P. copsychi* n. sp. possess a longer oesophagus when compared to the remaining three species. In addition, the oesophagus is clearly divided into a muscular and glandular portion in the new species, but not in

Table 1
Comparative morphometric data for *Pelecitus copsychi* n. sp. and congeners recorded from avian hosts in Indomalaya and Australia

Species	*Pelecitus copsychi* n. sp.[a]	*Pelecitus ceylonensis* Dissanaike, 1967	*Pelecitus galli* Dissanaike & Fernando, 1974	*Pelecitus bartneri* Spratt, 2010
Host	*Copsychus malabaricus* (Muscicapidae)	Ash dove (Columbidae), chicken (Phasianidae), crow (Corvidae)	*Gallus gallus spadiceus* (Phasianidae)	*Psephotus chrysopterygius* (Psittacidae)
Locality	Sungai Teris, Pahang, Malaysia	Colombo, Sri Lanka	Tasek Bera, Pahang, Malaysia	Brisbane, Queensland, Australia
Reference	Present study	Dissanaike (1967)	Dissanaike & Fernando (1974)	Spratt (2010)
Female	($n = 8$)			
Body length (mm)	8.0 (4.5–8.7)	6.0–9.6	15.6–20.4	13.3–15.5
Body width at midbody	570 (390–570)	320–460	550–650	530–901
Nerve-ring from anterior extremity	193 (168–213)	120–155	170–190	159–186
Vulva from anterior extremity	420 (330–500)	335–440	670–780	594–1113
Muscular oesophagus length	450 (375–610)	Not divided	Not divided	280–345
Glandular oesophagus length	570 (530–800)	–	–	450–504
Oesophagus length	1020 (1020–1250)	580–700	850–870	797 (mean)[b]
Tail length	138 (80–138)	50–88	150–180	150–169
Microfilaria	($n = 10$)			
Body length	158–183	190–260	180–295	207–212
Body width	3–5	5–6	6.7–8.2	4–5
Male	($n = 4$)			
Body length (mm)	4.6 (4.5–4.6)	3.55–5.3	9–11	5.9–7.3
Body width at midbody	350 (280–380)	185–300	400–500	344–466
Nerve-ring from anterior extremity	175 (163–175)	125–150	140–160	158–170
Muscular oesophagus length	480 (310–600)	Not divided	Not divided	260–292
Glandular oesophagus length	670 (650–710)	–	–	345–413
Oesophagus length	1150 (980–1150)	460–600	720–825	641 (mean)[b]
Left spicule length	80 (80–90)	68–82	85–95	83–94
Right spicule length	73 (62–73)	55–70	70–90	67–77
Tail length	55 (55–68)	42–55	40–48	48–58

[a] Measurements of the holotype female and allotype male of *Pelecitus copsychi* n. sp. are present first, followed by the range, including the type-specimen, in parentheses.
[b] Spratt (2010).

P. ceylonensis or *P. galli*. While in the present specimens, the vulva is located at the level of the anterior half of the oesophagus (Fig. 2A), it is located at the level of the posterior half in the remaining species (Table 1). Tail length of the present females is greater than that of *P. ceylonensis*, but shorter than that of *P. galli* and *P. bartneri*. Furthermore, the microfilariae of *P. copsychi* n. sp. are somewhat shorter than those of the remaining species.

The present species can be further differentiated from *P. galli* in that its males are shorter and more slender at midbody, but with a tail that is longer than that of *P. galli*. In *P. copsychi* n. sp., granular or hyaline inclusions were not seen in the caudal alae of males, but such inclusions are present in the caudal alae of *P. bartneri* (see Spratt, 2010).

Pelecitus fulicaeatrae has a wide host and geographical range. The species has been recorded in *Fulica atra* L. (Gruiformes) and various other hosts from England, Germany, Russia, Japan, France, Australia, Canada and Madagascar (Supplementary Table S1). However, *P. copsychi* n. sp. can be readily distinguished from this cosmopolitan species in that in *P. fulicaeatrae*, the pre-oesophageal ring is delicate, the oesophagus is not divided, and the vulva is situated at the level of the posterior half of the oesophagus, close to or posterior to the oesophago-intestinal junction (Vanderburgh et al., 1984; Bartlett & Greiner, 1986; Spratt, 2010).

The present species is further distinguished from the remaining 13 congeners parasitizing avian hosts by a combination of characters (Bartlett & Greiner, 1986; Bartlett & Anderson, 1987a). With the exception of *Pelecitus vuylstekae* Vuylsteke, 1957 and *P. copsychi* n. sp., in which the vulva in females is at the level of the anterior half of the oesophagus, the vulva is positioned at the level of the posterior half of the oesophagus in the remaining species. However, *P. vuylstekae* can be distinguished from the new species in that its females (19–25 *vs* 4.5–8.7 mm) and males (8–9 *vs* 4.5–4.6 mm) are longer, its spicules are not strongly sclerotized, the blade of the left spicule is slender, and inclusions are occasionally present in the caudal alae (Bartlett & Greiner, 1986). In the pre-oesophageal ring being delicate or absent, *Pelecitus andersoni* Bartlett & Greiner, 1986, *Pelecitus chabaudi* Bartlett & Greiner, 1986, *Pelecitus circularis* (Molin, 1860), *Pelecitus helecinus* (Molin, 1860), *Pelecitus polamaetus* Vuylsteke, 1957 and *Pelecitus tubercauda* Vanderburgh, Anderson & Stock, 1984 differ from the new species in which the pre-oesophageal ring is readily apparent (Bartlett & Greiner, 1986). In addition, the vulva is only slightly or not protuberant in *P. andersoni*, *P. chabaudi* and *P. helicinus*, while it is markedly protuberant in *P. copsychi* n. sp. Furthermore, caudal inclusions are present in *P. andersoni*, *P. helecinus*, *P. polamaetus* and *P. tubercauda* (see Bartlett & Greiner, 1986), but absent in the present specimens. Additional distinguishing features between these congeners and the new species are the oesophagus being undivided or only indistinctly divided in *P. chabaudi*, *P. circularis* and *P. tubercauda* and the weakly sclerotized spicules in *P. polamaetus*, with a narrow blade seen in the left spicule (Bartlett & Greiner, 1986), as opposed to a clearly divided oesophagus and well-sclerotized spicules with a broad left blade in *P. copsychi* n. sp. *Pelecitus anhingae* Vuylsteke, 1957 and *Pelecitus tercostatus* (Molin, 1860) are similar to the new species in that the pre-oesophageal ring is readily apparent and the oesophagus is divided (Bartlett & Greiner, 1986). However, in the former two species, the spicules are not well-sclerotized and the left spicule has a narrow blade (Bartlett & Greiner, 1986). A further congener in birds, *Pelecitus armenica* Chertkova, 1945 can be distinguished from the new species by females being longer (15.5 mm) and males having longer spicules (left spicule 150 μm and right spicule 110 *vs* 80–90 μm and 62–73 μm, respectively) (Bartlett & Greiner, 1986). Bartlett & Greiner (1986) considered *Pelecitus barusi* Coy Otero, 1982 morphologically very close to *P. galli*, but postponed a decision on their synonymy until more material of the former species could be examined. *Pelecitus barusi* differs from the new species in the females being larger (15–20 mm long) and the oesophagus being undivided or indistinctly divided (Bartlett & Greiner, 1986).

Little information is available on *P. major* and *P. spiralis* from Passeriformes in Nigeria. However, given their host spectrum (Ploceidae) and geographical distribution in Africa (Bartlett & Anderson, 1987a), it is unlikely that they should be conspecific with our material collected from a bird of the Muscicapidae in Malaysia.

Bartlett & Greiner (1986) transferred to *Pelecitus* two filarial species parasitic in mammals, previously placed in the genus *Dirofilaria*: *Pelecitus*

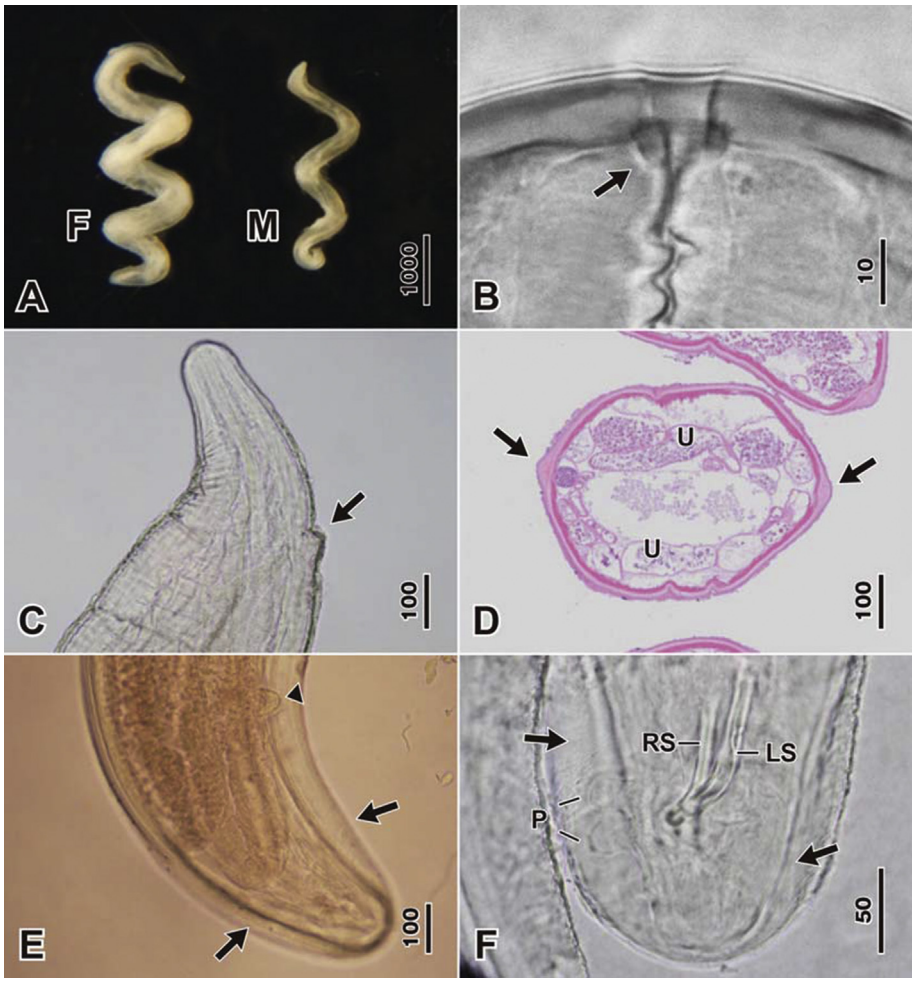

Fig. 1. Light micrographs of *Pelecitus copsychi* n. sp. **A** Corkscrew-shaped body of female (F) and male (M). **B** Pre-oesophageal cuticular ring (arrow) of female. **C** Anterior part of female, lateral view showing the protruding vulva (arrow). **D** Transverse section of female at midbody showing the lateral alae (arrows) and uteri with microfilariae (U) (HE staining). **E** Posterior part of female, ventral view showing post-deirid (arrowhead) and lateral alae (arrows). **F** Posterior part of male, ventral view showing lateral alae (arrows), pedunculate papillae (P), left spicule (LS) and right spicule (RS). Scale-bars are in micrometres.

roemeri (von Linstow, 1905) in the knee region of *Macropus giganteus* Shaw (Macropodidae) in Australia and *Pelecitus scapiceps* (Leidy, 1886) in the ankle region of *Sylvilagus floridanus* (Allen) (Leporidae) in North America. Later, *P. meridionaleporinus* was described from the subcutaneous tissue at the ears of *L. flavigularis* in Mexico (Jiménez-Ruiz et al., 2004). *Pelecitus roemeri* can be clearly distinguished from the new species in that its females (23–110 mm long; from *M. giganteus*) are larger, its spicules are not strongly sclerotized and the left blade is slender (Bartlett & Greiner, 1986; Jiménez-Ruiz et al., 2004; Spratt, 2011). Contrary to *P. copsychi* n. sp., in *P. scapiceps* the pre-oesophageal ring is not readily apparent, the oesophagus is indistinctly divided, and the vulva is situated posterior to the oesophago-intestinal junction. In addition, females of *P. scapiceps* are larger (25–30 mm long) (Sonin, 1975; Bartlett & Greiner, 1986; Jiménez-Ruiz et al., 2004). *Pelecitus meridionaleporinus* differs from the present species in having larger females (12.5–28 mm long) and microfilariae (210–285 *vs* 158–183 μm long) as well as males with longer spicules (left spicule102–119 *vs* 80–90 μm, right spicule 75–102 *vs* 62–73 μm) (Jiménez-Ruiz et al., 2004).

Based on the morphological and morphometric differences outlined above, we consider the present specimens as separate from their three congeners in Indomalaya and Australia as well as from the remaining 17 congeners in avian and mammalian hosts worldwide (Supplementary Table S1).

3.2. Molecular identification

A p-distance threshold has to be established to conclude if two sequences represent the same or different species. The optimum threshold (OT) is the p-distance value minimizing the false-positive and false-negative errors in the intraspecific and interspecific assignments (Ferri et al., 2009). Owing to the lack of intraspecific data in the present dataset, the OT could not be estimated. However, Ferri et al. (2009) previously estimated an OT for the *cox*1 marker of the Onchocercidae. Their p-distance threshold, set at 4.8%, was used in the present study. The mean interspecific distance established for the present dataset was 14.67% (S.E. = 2.15%; range = 2.76–23.03%), which falls within the range of other similar studies (Ferri et al., 2009; Lefoulon et al., 2017).

The calculated p-distance for *cox*1 gene sequences between *P. copsychi* n. sp. and closely related species was 11.2% for *F. candezei*, 11.2% for *L. loa* and 14.1% for *P. fulicaeatrae*. The genetic variation between the present two specimens (ID nos. 168-4 and 268-5) obtained from the swollen footpad of the host's left leg was 0.4%. In *Onchocerca* spp., *cox*1 intraspecific genetic distance is lower than 2% (Lefoulon et al., 2017). Therefore, our molecular results corroborate that *P. copsychi* n. sp. is distinct from *P. fulicaeatrae* at the species level, whereas the genetic variation between the two specimens from *C. malabaricus* corresponds to intraspecific differences.

3.3. Molecular phylogeny

Overall, the concatenated phylogeny was well resolved and strongly supported. The Bayesian inference analysis resulted in a tree with five monophyletic Onchocercidae clades (ONC1–5) as previously proposed by Lefoulon et al. (2015). Most genera included in this study appeared as monophyletic (Fig. 3). The subfamily Dirofilariinae was divided into two clades: the genus *Dirofilaria* was placed in ONC3, whereas *Pelecitus*,

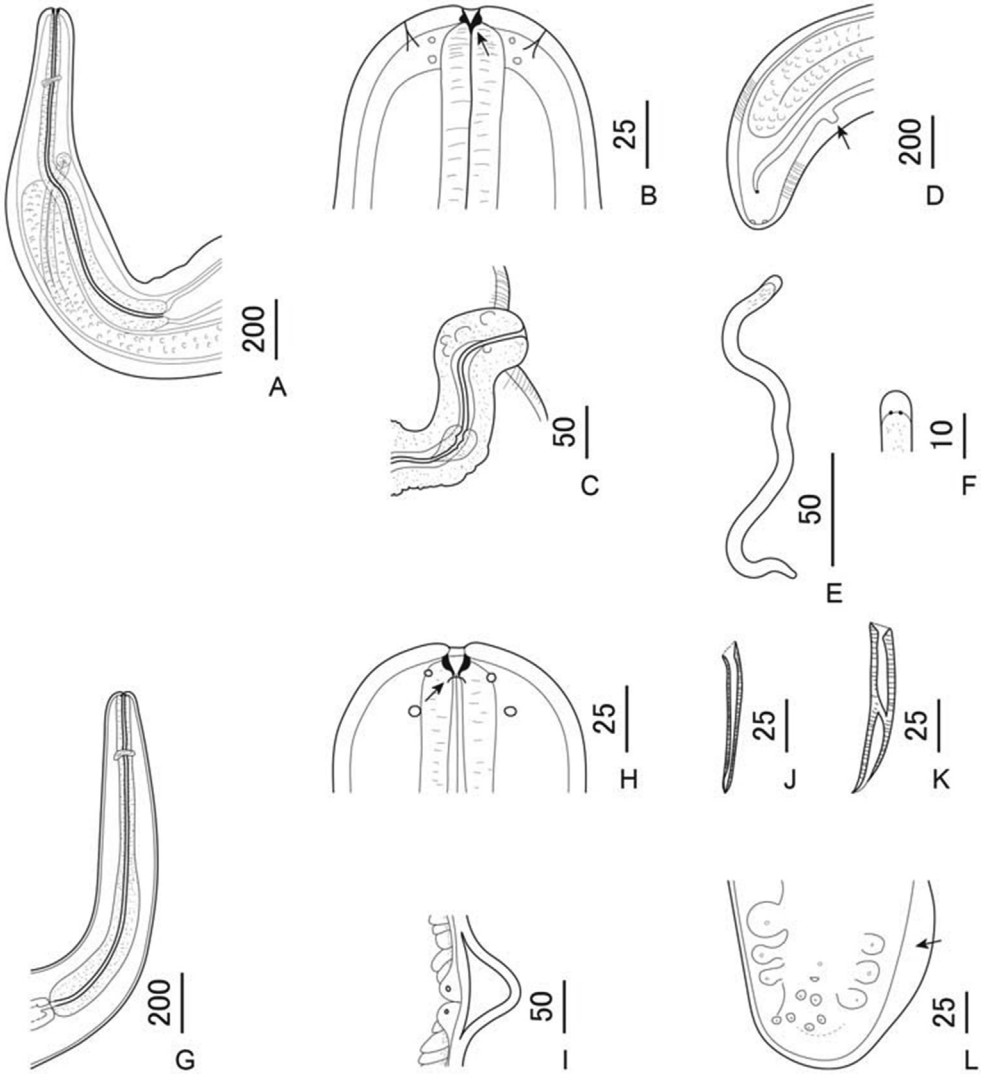

Fig. 2. Line drawings of *Pelecitus copsychi* n. sp. Females (**A–D**), microfilariae (**E**, **F**) and males (**G–L**). **A** Anterior region, right sublateral view. **B** Anterior extremity, dorsoventral view. Note the two pairs of cephalic papillae and amphids and the preoesophageal cuticular ring (arrow). **C** Protruding vulva, right lateral view. **D** Posterior extremity, ventral view with post-deirid arrowed. **E** Microfilaria with sheath and blunt tail. **F** Head with loose sheath. **G** Anterior region, lateral view. **H** Anterior extremity, lateral view. Note amphid (arrow). **I** Lateral ala at posterior end. **J** Right spicule, dorsoventral view. **K** Left spicule, dorsoventral view. **L** Male posterior extremity, ventral view. Note caudal ala (arrow). Scale-bars are in micrometres.

including *P. copsychi* n. sp., *Foleyella* Seurat, 1917 and *Loa* Stiles, 1905 were placed in ONC5 (Fig. 3). *Pelecitus* is the only genus in this clade whose members parasitize birds and mammals, while species of *Foleyella* infect squamates and *L. loa* only infects humans (Bartlett, 1986; Bain et al., 1998). Notably, the genus *Pelecitus* presented as paraphyletic in that *P. copsychi* n. sp. together with *F. candezei* formed a well-supported clade as sister group to *P. fulicaeatra* within the Bayesian tree (Fig. 3). Affiliations within the ML phylogeny were similar to those in the Bayesian tree, although deeper nodes were less strongly supported (Supplementary Figure S1).

3.4. *Wolbachia* detection

Following immunohistological staining, *Wolbachia* endosymbionts could neither be detected in the genital system nor in the lateral chords of sections of a female *P. copsychi* n. sp. Similarly, nested PCR analysis failed to detect the presence of *Wolbachia* in the two specimens screened.

4. Discussion

Bartlett (1986) and Bartlett & Anderson (1987b) stated that although species of *Pelecitus* from birds exhibit a close morphological resemblance to *Foleyella* from squamates, many species of *Pelecitus* have a corkscrew-shaped body, whereas all adults of *Foleyella* spp. are straight or gently curved. Consequently, based on the corkscrew-shaped body in adults of both sexes as well as the predilection site in the host and the host taxon itself, we assigned the present specimens to *Pelecitus*.

Bain et al. (1993) suggested that the anterior position of the vulva in *Onchocerca ramachandrini* Bain, Wahl & Renz, 1993 from *Phacochoerus africanus* (Gmelin) (Suidae: Phacochoerinae) in the Afrotropical realm represented one of the ancestral characteristics within members of the genus *Onchocerca* Diesing, 1841, while a short, undivided oesophagus was considered an evolved character. In the Oswaldofilariinae Chabaud & Choquet, 1953, a long oesophagus was viewed as an ancestral characteristic (Chabaud & Bain, 1994). If these hypotheses are correct, we propose that *P. copsychi* n. sp. has some ancestral characteristics in comparison to its congeners *P. ceylonensis*, *P. galli* and *P. bartneri* from Indomalaya and Australia. The position of the vulva is anterior in *P. copsychi* n. sp., situated at the level of the anterior half of the oesophagus, not the posterior half, and the oesophagus is longer. In addition, the oesophagus is divided in the new species (as well as in *P. bartneri*), but undivided in *P. ceylonensis* and *P. galli* (Table 1).

With regard to the geographical distribution of *Pelecitus* spp. from birds, many species have been recorded in Africa and South America and only three species have been reported to date from Indomalaya and Australia. In the present study, we describe *P. copsychi* n. sp. from *C. malabaricus* in the primary forest of Pahang, Peninsular Malaysia. This bird has not been listed as host of previously described species of *Pelecitus* (see Bartlett & Greiner, 1986; Spratt, 2011; Supplementary Table S1), and thus constitutes a new host record for this genus.

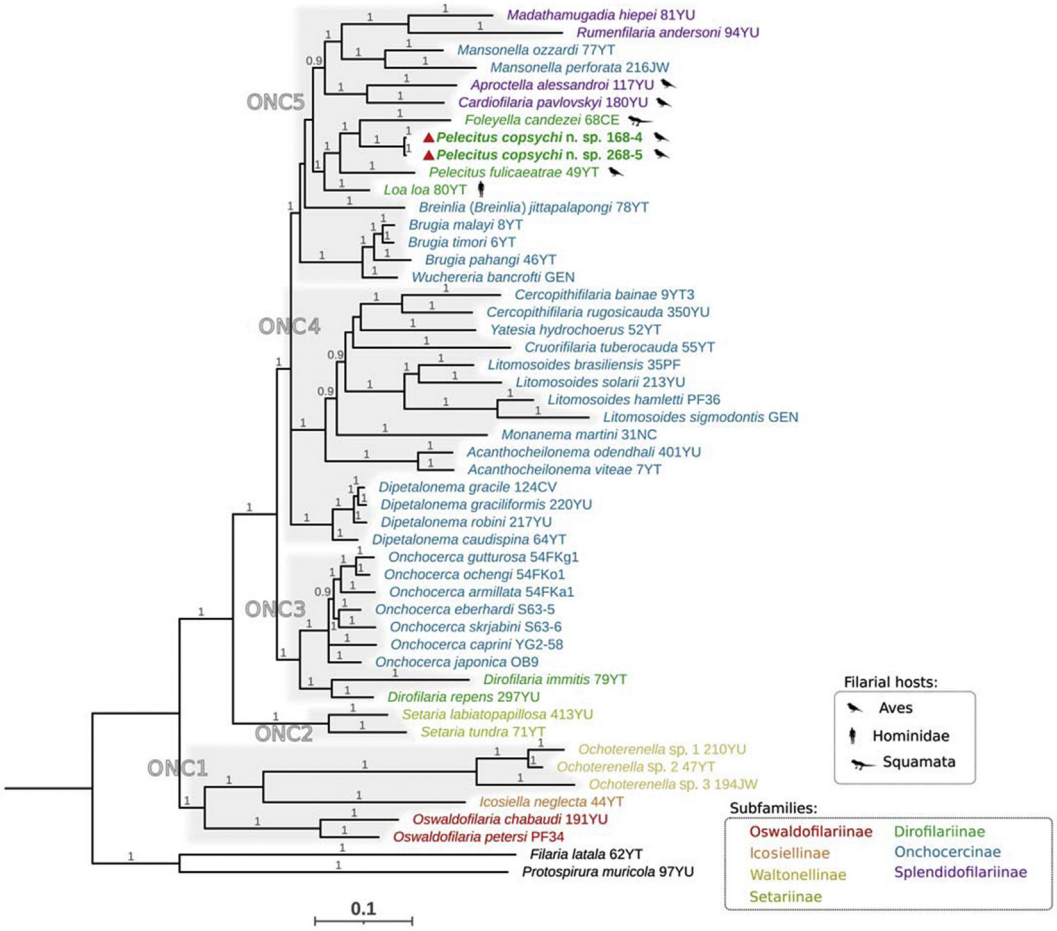

Fig. 3. Clades of the Onchocercidae (ONC1–5) based on partitioned concatenated datasets of *12S* rDNA, *cox*1, *rbp1*, *hsp70*, *myoHC*, *18S* rDNA and *28S* rDNA sequences. The total length of the dataset is *c.*3979 bp. Fifty onchocercid sequences (representing 49 species) were analysed. *Filaria latala* and *Protospirura muricola* were used as the outgroups. The topology was inferred using Bayesian inference on two runs of five million generations, with the first 25% of the trees removed as "burn-in". 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.

Copsychus malabaricus is distributed in the southern Thai-Malay Peninsula (Rasmussen & Anderson, 2005). Although *P. copsychi* n. sp. was discovered in close geographical vicinity to *P. galli*, its host, *C. malabaricus*, belongs to the order Passeriformes, whereas the host of *P. galli*, *G. g. spadiceus*, belongs to the order Galliformes (Dissanaike & Fernando, 1974).

Microfilariae were not found in the blood smears of *C. malabaricus*, suggesting skin-inhabiting microfilariae. According to Bartlett & Anderson (1987b), the skin-inhabiting microfilariae of *P. fulicaeatrae* are extraordinarily rare, and skin-inhabiting microfilariae have not previously been reported for any of the more than 140 known species of avian filariae. In future studies, skin snips should be taken from birds to test this hypothesis.

Regarding zoonotic infections, Bain et al. (2011) suggested that an intraocular worm from a patient in Brazil belonged to the genus *Pelecitus*. Similarly, an intraocular worm from a patient in Colombia was originally identified as *Loaina* sp. (Botero et al., 1984), but Bartlett & Greiner (1986) considered it more likely that the worm belonged to a species of *Pelecitus* parasitic in birds. Bain et al. (2011) supported their opinion. While we conclude that *Pelecitus* spp. of avian origin may be of zoonotic importance in South America, evidence of the zoonotic potential of the four *Pelecitus* spp. (including *P. copsychi* n. sp.) from avian hosts in Indomalaya and Australia has yet to be found. Bartlett & Greiner (1986) suggested that *Pelecitus* spp. of mammals represent "capture" of avian filariae by mammals. In other words, *Pelecitus* spp. in mammals could have originated from avian filariae through host-switching events facilitated by vectors.

The vector of *P. ceylonensis* in birds is a mosquito, namely *Coquillettidia crassipes* (van der Wulp) (syn. *Mansonia crassipes* (van der Wulp)) (Diptera) (Dissanaike, 1967), and development of *P. fulicaeatrae* was recorded in the chewing louse *Pseudomenopon pilosum* (Scopoli) (Mallophaga) (Bartlett & Anderson, 1987b). In mammalian hosts, the vectors of *P. scapiceps* are mosquitoes and those of *P. roemeri* tabanids (Highby, 1943; Spratt, 1972). Therefore, *Pelecitus* species possess the most heterogeneous vector range within the Onchocercidae. Although we did not find any potential vectors for *P. copsychi* n. sp. in the present study, we speculate that, like its congeners, the new species is transmitted by haematophagous arthropods.

The division of the subfamily Dirofilariinae is not only well-supported from a molecular standpoint (Lefoulon et al., 2015; this paper), but can also be observed in certain biological particularities. First, the third moult occurs as early as day 2–3 post-inoculation in *Dirofilaria* spp. (similar to *Onchocerca* spp.), but at a later stage, approximately one week post-inoculation, in the remaining genera of this subfamily (Bain et al., 1998). Secondly, the genus *Dirofilaria* shares morphological characteristics with the genus *Onchocerca*: buccal capsule normally developed in infective larvae but reduced in adults; a cylindrical tail with rounded extremity and tiny caudal lappets in infective larvae (Bain et al., 2008).

Based on the redescriptions of *F. candezei* and *Foleyella furcata* (von Linstow, 1899), Bartlett (1986) suggested that the close morphological resemblance between *Foleyella* and *Pelecitus* indicates their close phylogenetic affinities. The present phylogenetic analysis revealed *Pelecitus* as

paraphyletic (Fig. 3). Two hypotheses may be put forward. *Pelecitus* may have a complex evolutionary history, possibly owing to its worldwide geographical distribution and a broad host spectrum at genus level (11 orders, representing more than half of the avian orders and two mammalian families) (Bartlett & Greiner, 1986; Jiménez-Ruiz et al., 2004). Alternatively, the current limited set of molecular data (two species out of 21 known species, including *P. copsychi* n. sp.) might not be inclusive enough to support reliable conclusions about the phylogenetic relationships of this genus. Adding molecular data for species of *Pelecitus* representing a wider host spectrum and geographical range may in future provide clarification. In the present phylogeny, *Pelecitus* formed a clade with *Foleyella* and *Loa* (second clade of ONC5). This clade presents the most heterogeneous host range within all onchocercid clades, comprising reptiles, birds and mammals.

Regarding the phylogenetic relationships between *Wolbachia* supergroups and clades of the Onchocercidae, an ancestral absence of *Wolbachia*, horizontal transfer events, secondary losses and local coevolution with host filariae have been discussed (Bain et al., 2008; Ferri et al., 2011; Lefoulon et al., 2012, 2016; Uni et al., 2020). In this study, neither immunohistochemical staining nor molecular screening detected *Wolbachia* endosymbionts in *P. copsychi* n. sp. Bain et al. (2008) and Lefoulon et al. (2016) suggested that ancestral filarial subfamilies such as Setariinae Yorke & Maplestone, 1926, Waltonellinae Bain & Prod'hon, 1974 and Oswaldfilariinae have no acquisition of *Wolbachia*, whereas Dirofilariinae and Onchocercinae Leiper, 1911 acquired *Wolbachia* supergroup C. Subsequently, secondary losses occurred in *P. fulicaeatrae*, *F. candezei* and *L. loa* in the Dirofilariinae; as well as in *Onchocerca flexuosa* (Wedl, 1856), *Acanthocheilonema* spp., *Cercopithifilaria* spp. (except for *Cercopithifilaria japonica* (Uni, 1983) with *Wolbachia* supergroup F (Ferri et al., 2011)) and some other species in the Onchocercinae. We attribute the absence of *Wolbachia* endosymbionts in *P. copsychi* n. sp. to a secondary loss.

To date, five species of avian filariae have been tested for *Wolbachia* endosymbionts and have been found *Wolbachia*-free: *Chandlerella quiscali* (Linstow, 1904), *A. alessandroi* and *C. pavlovskyi* in the Splendidofilariinae Chabaud & Choquet, 1953 well as *P. fulicaeatrae* and *P. copsychi* n. sp. in the Dirofilariinae (McNulty et al., 2012; Lefoulon et al., 2016; this study). One might hypothesize that avian filariae in general are devoid of *Wolbachia*. However, compared to the total number of filarial species infecting birds (*c.*170 according to Bartlett (2008)), the number of tested species is still too limited to support a reliable conclusion. In addition, *L. loa* and *F. candezei* do not harbour any *Wolbachia* endosymbionts either (McGarry et al., 2003; Lefoulon et al., 2016), suggesting that this entire sub-clade within ONC5 may be free of *Wolbachia* (Fig. 3; Supplementary Figure S1).

5. Conclusions

Pelecitus copsychi was described as a new species from *C. malabaricus* in Peninsular Malaysia, integrating morphological as well as molecular characteristics. The filariae occurred free in the footpad of the host and caused swelling at the site of infection. *Copsychus malabaricus* represents a new host record for the genus *Pelecitus*. Phylogenetic analysis based on seven genes (two mitochondrial genes, *12S* rDNA and *cox*1; and five nuclear genes, *18S* rDNA, *28S* rDNA, *MyoHC*, *rbp1* and *hsp70*), revealed the new species as closely related to *P. fulicaeatrae* from birds and *F. candezei* from squamates in Africa. However, *cox*1 analysis supported the new species as being distinct from *P. fulicaeatrae* at the species level. In the Bayesian and maximum-likelihood inferences, *P. copsychi* n. sp. clustered with other members of the Dirofilariinae as well as some genera of the Onchocercinae and Splendidofilariinae in a well-supported clade named ONC5, sister to all other taxa evaluated within the Onchocercidae (Lefoulon et al., 2015). The present study corroborates the division of the Dirofilariinae into two distinct groups, linking *Dirofilaria* spp. on one side and *P. fulicaeatrae*, *P. copsychi* n. sp., *F. candezei* and *L. loa* on the other side.

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, Malaysia.

CRediT author statement

Shigehiko Uni, Ahmad Syihan Mat Udin, Jules Rodrigues, Coralie Martin, Kerstin Junker: conceptualization, data curation, formal analysis, funding acquisition, methodology, resources, writing - original draft, writing - review & editing. Poai Ean Tan, Takeshi Agatsuma, Van Lun Low, Yvonne Ai-Lian Lim, Weerachai Saijuntha, Hasmazaiti Omar, Nur Afiquah Zainuri, Masako Fukuda, Daisuke Kimura, Makoto Matsubayashi, Shoji Uga, Hiroyuki Takaoka, Mohd Sofian Azirun, Rosli Ramli: methodology, resources, writing - review & editing. All authors read and approved the final manuscript.

Data availability

The newly generated gene sequences are deposited in the GenBank database under the accession numbers OK480041, OK480043 (*cox*1), OK480976-OK480977 (*12S* rRNA), OK481072-OK481073 (*18S* rRNA), OK493401-OK493402 (*MyoHC*), OK493398-OK493399 (*hsp70*), OK493403-OK493404 (*rbp1*) and OK481079-OK481080 (*28S* rRNA) for *P. copsychi* n. sp. Type-material is deposited in the MNHN, Paris, France, under accession numbers MNHN-IN-107YT for the holotype female of *P. copsychi* n. sp. and MNHN-IN-108YT for the allotype male. The paratypes are deposited in the Institute of Biological Sciences, Universiti Malaya, Malaysia, under accession numbers Pf8-1–7 and Pm8-9–11.

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.

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 the late Dr Lim Boo Liat and the staff of the Department of Wildlife and National Parks, Malaysia, who conducted the Scientific Inventory in Sungai Teris, Krau Wildlife Reserve.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2022.100078.

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