RAFFLES BULLETIN OF ZOOLOGY **63**: 49–65
Date of publication: 27 February 2015
http://zoobank.org/urn:lsid:zoobank.org:pub:D9C2448D-4BAE-4F9E-9C1E-36CF02994A63

Three new species of *Ligophorus* Euzet & Suriano, 1977 (Monogenea: Ancyrocephalidae) from *Moolgarda buchanani (*Bleeker) off Johor, Malaysia based on morphological, morphometric and molecular data

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Abstract. *Ligophorus liewi*, new species, *L. johorensis*, new species, and *L. grandis*, new species, are described from *Moolgarda buchanani* off Johor, Malaysia. *Ligophorus liewi*, new species, is similar to *L. kederai* in having fenestrated ventral anchors but differs in having a distally tapered and bent accessory piece of the copulatory organ. *Ligophorus johorensis*, new species, is similar to *L. kedahensis* but it differs in having a raised antero-median protuberance (AMP) and longer copulatory tube. *Ligophorus grandis*, new species, is the largest Malaysian *Ligophorus* species and can be differentiated from the other species by its distally hooked-shaped accessory piece. Principal Component Analysis (PCA) scatterplot indicates that these three new species can be differentiated from each other and 10 described Malaysian *Ligophorus* species based on the morphometries of their sclerotised hard parts. Numerical taxonomy (NT) analysis indicates that the three new species are 38% similar to each other and the other 59 described *Ligophorus* species with differences primarily observed in size and shape of anchors, bars and male copulatory organs. Partial 28S rDNA and complete ITS1 from the three new and 10 previously described Malaysian *Ligophorus* species were sequenced. Maximum parsimony (MP) analysis based on these molecular data recovered a monophyletic clade that includes the three new species, and three previously described species, *L. kedahensis*, *L. fenestrum* and *L. kederai* from *M. buchanani*.

Key words. morphology, morphometry, Mugilidae, PCA, numerical taxonomy, 28S rDNA, ITS1

INTRODUCTION

Monogenean worms are ectoparasitic flatworms found primarily on dermal layers and gills of fish hosts. The genus *Ligophorus*, with 59 species, are found in 17 mullet host species throughout the Pacific and Atlantic Oceans (Sarabeev et al., 2013; Soo & Lim, 2012, 2015; El-Hafidi et al., 2013a, b; Kritsky et al., 2013). Of these, 10 species (*L. parvicopulatrix* Soo & Lim, 2012, *L. bantingensis* Soo & Lim, 2012, *L. chelatus* Soo & Lim, 2012, *L. navjotsodhii* Soo & Lim, 2012, *L. funnelus* Soo & Lim, 2012, *L. careyensis* Soo & Lim, 2012, *L. belanaki* Soo & Lim, 2015, *L. kedahensis* Soo & Lim, 2012, *L. fenestrum* Soo & Lim, 2012 and *L. kederai* Soo & Lim, 2015) were collected from two mullet species, *Liza subviridis* (Valenciennes, 1836) and *Moolgarda buchanani* (Bleeker, 1853), in Malaysian waters (Soo & Lim, 2012, 2015).

In a recent survey of the monogenean worms on the host *M. buchanani* from the Straits of Johor, we recovered three described (*L. kedahensis*, *L. fenestrum* and *L. kederai*) and three new *Ligophorus* species. We thus aim to describe these three species and determine their relationships to congeners based on partial 28S rDNA and complete ITS1 sequences.

MATERIAL AND METHODS

Collection of host and parasites. *Moolgarda buchanani* was collected from the southern waters off Johor, Peninsular Malaysia (01°2’6”N 103°5’4”E) in November 2013 and frozen. The gills were then excised and gently scraped to dislodge monogenean worms, the latter were then transferred using a fine pipette to a small drop of water onto a clean slide. A clean coverslip was placed over the water droplet and its four corners sealed with clear nail varnish. Ammonium-picrate-glycerine (5:1 mixture of 40% formalin and glycerine mixed with one part of a saturated aqueous solution of picric acid) was added underneath the coverslip to fix and clear the monogeneans (Lim, 1991). Images of the hard anatomical structures of the *Ligophorus* species were captured using a Leica digital camera and an image analysis software (QWin Plus). The hard parts of the haptor and copulatory organs were illustrated using a digitising tablet (WACOM) and Adobe Illustrator software (version CS6). These specimens were then washed and dehydrated through a graded ethanol series and mounted in Canada Balsam without staining (Lim, 1991). Type specimens of

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ISSN 2345-7600 (electronic) | ISSN 0217-2445 (print)

the three new species were deposited at the Natural History Museum, London, United Kingdom (NHMUK), Zoological Reference Collection, Lee Kong Chian Natural History Museum, National University of Singapore, Singapore (ZRC) and Zoological Museum University of Malaya, Kuala Lumpur (MZUM). The following museum specimens were examined as comparative material: paratype *Ligophorus kederai* MZUM(P)2013.37(P)–MZUM(P)2013.40(P); paratype *Ligophorus kedahensis* MZUM(P)2013.60(V)–MZUM(P)2013.65(V); paratype *Ligophorus fenestrum* MZUM(P)2013.199(V) – MZUM(P)2013.203(V); paratype *Ligophorus bykhowskyi* Dmitrieva, Gerasev, Gibson, Pronkina & Galli, 2012, BMNH No. 2011.11.17.1–4; paratype *Ligophorus simpliciformis* Dmitrieva, Gerasev, Gibson, Pronkina & Galli, 2012, BMNH No. 2011.11.17.5–11; paratype *Ligophorus bipartitus* Dmitrieva, Gerasev, Gibson, Pronkina & Galli, 2012, BMNH No. 2011.11.17.12–13; paratype *Ligophorus mamaevi* Dmitrieva, Gerasev, Gibson, Pronkina & Galli, 2012, BMNH no. 2011.11.17.24–26; paratype *Ligophorus surianoae* Dmitrieva, Gerasev, Gibson, Pronkina & Galli, 2012, BNMH no. 2011.11.17.2932.

Morphometrics and principal component analysis (PCA). PCA was used to affirm the status of the three new *Ligophorus* species based on morphometric data. The diagnostic sclerotised hard parts of 92 specimens belonging to the three new *Ligophorus* species were measured using the Leica QWin software, following Soo & Lim (2012). A total of 18 parameters were measured (Table 1): 10 from dorsal and ventral anchors (inner root, outer root, inner length, outer length and point), one from the marginal hook (hook length), three from the ventral bar (length, width and distance between lateral pieces of the antero-median protuberance or AMP), two from dorsal bar (length and width) and two from the male copulatory organ (length of copulatory tube and length of accessory piece). All measurements are presented as the mean, followed by its range within parentheses, in micrometers (µm) and used in the description and differential diagnoses of the new species. Morphometric data from these 92 specimens of the three new species and 373 specimens of 10 *Ligophorus* species previously described by Soo & Lim (2012; 2015) were analysed using PCA in R (version 3.1.0; R Core Development Team 2014).

Table 1. Morphometrics (µm) of three new *Ligophorus* species obtained from *Moolgarda buchanani*; n = number of specimens measured and range in size given in brackets.

Species Parameters	***L. liewi*, new species (n = 31)**	***L. johorensis*, new species (n = 39)**	***L. grandis*, new species (n = 22)**
Body length	1064 (823–1270)	1124 (955–1304)	1966 (1355–2167)
Body width	128 (90–166)	175 (131–214)	321 (200–379)
Haptor length	91 (51–144)	89 (64–118)	96 (74–105)
Haptor width	175 (118–214)	200 (148–235)	145 (123–159)
Marginal hook	12 (10–13)	12 (11–13)	12 (10–13)
Ventral anchor:			
Inner root	18 (12–21)	19 (17–21)	20 (18–22)
Outer root	11 (7–15)	13 (10–14)	12 (8–14)
Inner length	48 (45–51)	40 (37–43)	41 (39–43)
Outer length	48 (45–49)	39 (35–42)	39 (37–42)
Point	11 (9–12)	9 (8–10)	10 (8–10)
Dorsal anchor:			
Inner root	19 (18–22)	18 (15–21)	21 (18–23)
Outer root	8 (6–11)	9 (5–10)	10 (7–12)
Inner length	48 (45–50)	38 (35–41)	44 (42–45)
Outer length	43 (41–45)	34 (28–38)	35 (34–37)
Point	12 (10–14)	12 (10–13)	11 (10–13)
Ventral bar:			
Width	48 (44–52)	51 (40–55)	45 (43–49)
Height	9 (6–12)	9 (6–13)	12 (9–14)
AMP width	8 (6–10)	9 (6–11)	3 (1–4)
Dorsal bar:			
Width	48 (54–63)	59 (52–74)	54 (51–57)
Height	6 (5–8)	5 (2–7)	8 (6–9)
Copulatory tube length	81 (71–88)	53 (46–60)	59 (52–63)
Accessory piece length	36 (29–42)	30 (25–37)	34 (31–40)

Numerical taxonomy (NT) analysis (Sneath & Sokal, 1973). NT analysis was used to determine how the three new *Ligophorus* species are related to each other and to the 59 described *Ligophorus* species, based on morphological and categorised metric characters. Morphological and metric data from *Ligophorus* species were collected following Soo & Lim (2012). A total of 59 character states representing the hard parts (anchor, bar and male copulatory organ) were identified (Table 2) and assigned to all species. A data matrix based on Jaccard's Index of Similarity was generated and neighbour-joining method was used to cluster the 62 species in the form of a dendrogram. The calculation of Jaccard's Index of Similarity and clustering were done in R (version 3.1.0; R Core Development Team 2014).

DNA extraction, PCR and DNA sequencing. The monogenean worms were removed from the gills of the hosts and preserved in 75% ethanol and identified. Genomic DNA was then extracted using DNEasy extraction kit from QIAGEN. The extracted DNA (5 µl) was used as a template in the PCR reaction to amplify the partial D1-D2 domain of the 28S rDNA, using forward primers C1 (5'-ACCCGCTGAATTTAAGCAT-3') (Hassouna et al., 1984) and reverse primer D2 (5' - TGGTCCGTGTTTCAAGAC-3') (Qu et al., 1988). The PCR reaction (50 µl) was performed in 1.5 mM $MgCl_2$, PCR buffer (Fermentas), 200 µM of each deoxyribonucleotide triphosphate, 1.0 µM of each PCR primer and 1 U of Taq polymerase (Fermentas) in a thermocycler (Eppendorf Mastercycler) using the following conditions: an initial denaturation at 95°C for 4 minutes, followed by 35 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. To amplify the internal transcribed spacer (ITS1), the primers Lig18endF (5'-GTCTTGCGGTTCACGCTGCT-3') and Lig5.8R (5'-GATACTCGAGCCGAGTGATCC-3') (Blasco-Costa et al., 2012) were used and the following thermocycling procedure was applied: an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 94°C for 40 seconds, 56°C for 30 seconds and 72°C for 45 seconds, followed by a final extension at 72°C for 4 minutes. An aliquot (10 µl) from the amplicon for both regions were electrophoresed in a 1.3% agarose gel, stained with ethidium bromide and viewed under a UV illuminator. The remaining 40µl of each amplicon was purified using a DNA purification kit (QIAGEN) and subjected to automated DNA sequencing (ABI 3730 DNA Sequencer, First Base Laboratories) using the same primers used for PCR amplification. Partial 28S rDNA and complete ITS1 from 13 *Ligophorus* species off Malaysia were sequenced (Table 3).

Analyses of molecular data. Two sets of molecular data from *Ligophorus*, the partial 28S rDNA dataset (13 sequences of Malaysian species from present study and 16 sequences from GenBank^{TM}) and combined datasets of partial 28S rDNA and complete ITS1 sequences (28S+ITS1) (13 partial 28S and complete ITS1 sequences of Malaysian species from present study; 14 partial 28S and complete ITS1 sequences from the GenBank^{TM}) (see Table 3) were analysed with *Ergenstrema mugilis* Paperna, 1964 (from GenBank^{TM}) as the outgroup. These DNA sequences were edited and aligned with Clustal X (Thompson et al., 1997) using default parameters and verified/edited visually by BioEdit version 7.0.5.3 (Hall, 1999). In the current sequence alignment, there were 542 alignable positions containing 228 parsimony informative sites for the 28S dataset while for the 28S+ITS1 dataset, there were 351 parsimony informative sites from 851 alignable positions. Maximum parsimony (MP) trees were constructed for both datasets. Maximum parsimony analysis was performed using PAUP* 4.0 b10 (Swofford, 2002) where full heuristic searches were conducted with 10 random sequence additions and tree bisection-reconnection (TBR) branch swapping. Bootstrap procedure with 1000 replications was performed to assess the robustness of the inferred relationships. The MP trees were displayed and edited using TreeView 1.6.6 (Page, 1996).

The degree of similarities of the 29 *Ligophorus* species (13 Malaysian species and 16 from the GenBank^{TM}) based on partial 28S rDNA were calculated using BioEdit for comparison purposes (Table 4). The 29 *Ligophorus* species abbreviated as follows: *L. acuminatus* Euzet & Suriano, 1977 = Acu, *L. minimus* Euzet & Suriano, 1977 = Min, *L. imitans* Euzet & Suriano, 1977 = Imi, *L. heteronchus* Euzet & Suriano, 1977 = Het, *L. confusus* Euzet & Suriano, 1977 = Con, *L. szidati* Euzet & Suriano, 1977 = Szi, *L. angustus* Euzet & Suriano, 1977 = Ang, *L. macrocolpos* Euzet & Suriano, 1977 = Mac, *L. pilengas* Sarabeev & Balbuena, 2004= Pil, *L. llewellyni* Dmitrieva, Gerasev & Pronkina, 2007= Lle, *L. chabaudi* Euzet & Suriano, 1977 = Cha, *L. cephali* Rubtsova, Balbuena, Sarabeev, Blasco-Costa & Euzet, 2006 = Cep, *L. mediterraneus* Sarabeev, Balbuena & Euzet, 2005 = Med, *L. vanbenedenii* Euzet & Suriano, 1977 JN996802 (Ebro Delta) = Van, *L. vanbenedenii* Euzet & Suriano, 1977 DQ157655 (China) = VanC, *L. leporinus* (Zhang & Ji, 1981) Gusev, 1985 = Lep, *L. parvicopulatrix* Soo & Lim, 2012 = Par, *L. bantingensis* Soo & Lim, 2012 = Ban, *L. belanaki* Soo & Lim, 2015 = Bel, *L. careyensis* Soo & Lim, 2012 = Car, *L. chelatus* Soo & Lim, 2012 = Che, *L. navjotsodhii* Soo & Lim, 2012 = Nav, *L. funnelus* Soo & Lim, 2012 = Fun, *L. fenestrum* Soo & Lim, 2012 = Fen, *L. kedahenesis* Soo & Lim, 2012 = Keda, *L. kederai* Soo & Lim, 2015 = Kede, *L. grandis*, new species = Gra, *L. johorensis*, new species = Joh and *L. liewi*, new species = Lie.

RESULTS

Results from PCA, NT and molecular analyses are discussed prior to the descriptions of the three species so that information from these analyses can be incorporated into their diagnoses.

Clustering of *Ligophorus* species using PCA. The results of the PCA are presented in the form of a scatterplot (Fig. 1). Instead of the traditional presentation of plots, only the centroids and areas encompassed by the 13 *Ligophorus* species are given to avoid confusion due to the overlapping plots of several species. The 465 specimens of *Ligophorus* (10 previously described and present three new species) from off Peninsular Malaysia are separated into 13 clusters which correspond to the 10 known and three new *Ligophorus* species

Table 2. List of metric and morphological characters used in the numerical taxonomy (NT) analysis

Character	**Character State**
Copulatory organ	
Copulatory tube (length)	Long: >150 µm Medium: 81–149 µm Short: <80 µm
Type of initial part on copulatory tube	Simple lobe Ornamented lobe
Accessory piece (length)	Long: >90 µm (91–102 µm) Medium: 21–89 µm Short: <20 µm (15–20 µm) Connected to initial part Not connected to initial part
Type of accessory piece	Simple grooved tube Grooved tube with expanded distal part Grooved tube with expanded proximal part Two opposing pieces: one groove tube and one claw/hook-shaped piece Bifurcated ends with handle Bifurcated ends without handle Funnel Beak
Vagina:	Absent/Not observed Present Short: (15–29 µm) Medium: (30–99 µm) Long: (100–200 µm)
Ventral Bar:	
Width	Broad: >50 µm (51–78 µm) Narrow: ≤50 µm (9–50 µm)
Shape	Inverted curved bar Straight horizontal bar
Type of antero-median protuberance (AMP)	2 lateral non-membranous pieces and a raised median piece 2 lateral non-membranous pieces and a flat/depressed median piece 2 lateral membranous pieces and a raised median piece 2 lateral membranous pieces and a flat/depressed median piece Single bifurcated piece Single with bifurcating membranous pieces
Dorsal Bar	
Width	Broad: >50 µm (51–78µm) Narrow: ≤50 µm (9–50µm)
Shape	U-shape U-shape with shoulder V-shape V-shape with shoulder Broad V-shape Straight horizontal bar

Character	Character State
Ventral anchors	
Base	Parallel perforations present Parallel perforations absent
Inner Length	Long: ≥30 µm (30–49 µm) Short: <30 µm (14–29 µm)
Roots	Inner root longer than outer root Outer root longer than inner root Outer root = Inner root
Points	Long: ≥11 µm (11–18 µm) Short: ≤10 µm (1–10 µm)
Dorsal Anchors	
Base	Parallel perforations present Parallel perforations absent
Inner Length	Long: ≥30 µm (30–49 µm) Short: <30 µm (14–29 µm)
Roots	Inner root longer than outer root Outer root longer than inner root Outer root = Inner root
Points	Long: ≥11 µm (11–18 µm) Short: ≤10 µm (1–10 µm)

(Fig. 1). Based on the 18 parameters listed above, the 31 specimens of *L. liewi*, new species, are grouped closest to *L. fenestrum* whilst the 39 specimens of *L. johorensis*, new species, and the 22 specimens of *L. grandis*, new species, are grouped close together (Fig. 1). The first principal component axis (PC1, x-axis), which accounts for 41% of the total variations, is an index of the overall size of all the hard parts and it separates the 465 individuals into three groups as shown in the horizontal bar plot (Fig. 1). The second principal component (PC2, y-axis), which explains 38% of the total variation, is an index that contrasts the length of the copulatory tube and the width of both the dorsal and ventral bars against the other parameters, separating the *Ligophorus* specimens into three main groups, characterised by the peaks of the horizontal bar plot (Fig. 1).

Clustering of *Ligophorus* species using NT analysis. Based on the dendrogram from the NT analysis (Fig. 2), the 59 known and three new *Ligophorus* species are grouped into four main clusters at 60% similarity level and into 19 groups at the 95% similarity level. The dendrogram indicates that *L. liewi*, new species, *L. johorensis*, new species and *L. grandis*, new species, are grouped within the same clade based on the morphology of their anchors, bars and male copulatory organ (Fig. 2). *Ligophorus grandis*, new species, forms sister group with *L. johorensis*, new species, and *L. kedahensis* (Fig. 2).

Molecular analyses. The MP tree (Fig. 3A) constructed from 28S dataset consists of four clades. Clade 1 consists of two Malaysian species (*L. careyensis* and *L. belanaki*), 14 species from the Pacific Ocean (*L. minimus*, *L. acuminatus*, *L. macrocolpos*, *L. chabaudi*, *L. pilengas*, *L. llewellyni*, *L. heteronchus*, *L. cephali*, *L. mediterraneus*, *L. imitans*, *L. angustus*, *L. szidati*, *L. confusus* and *L. vanbenedenii* JN996802 from the type-host, *Liza aurata* (Risso, 1810) off Ebro Delta, Mediterranean Sea) and *L. leporinus* from China. Clade 2 consists of *Ligophorus* species found only in *M. buchanani* which include the three new species (*L. liewi*, new species, *L. johorensis*, new species and *L. grandis*, new species), *L. kedahensis*, *L. fenestrum* and *L. kederai* while *Ligophorus* species from *Liza subviridis* are grouped in Clade 3 (*L. bantingensis* and *L. parvicopulatrix*) and Clade 4 (*L. chelatus*, *L. navjotsodhii* and *L. funnelus*) (Fig. 3A). *Ligophorus vanbenedenii* DQ157655 from China is also found in Clade 3.

Ligophorus vanbenedenii DQ157655 (Wu et al., 2006) from *Liza cephalus* (Linnaeus, 1758) and *L. vanbenedenii* JN996802 (Blasco-Costa et al., 2012) from *Liza aurata* are shown to be in different clades (Fig. 3A). Further comparison of partial 28S rDNA sequence data reveals that *L. vanbenedenii* DQ157655 (from China) is only 88.9% similar to *L. vanbenedenii* JN996802 from *Liza aurata* (Mediterranean Sea) but 100% similar to *L. parvicopulatrix* from *L. subviridis* (from Malaysia) (Table 4). It is also noted that *L. leporinus* is grouped amongst the *Ligophorus* species (Fig. 3A) and is 99% similar to *L. belanaki* based on the similarities of partial 28S rDNA (Table 4) despite its position within the *Ligophorus* as '*incertae sedis*' by Sarabeev et al. (2013).

The MP tree (Fig. 3B) generated from the 28S+ITS1 dataset consists of two clades. Clade 1 consists of 14 species from the

Pacific Ocean (see above) while Clade 2 is made up of the current three new species and 10 described Malaysian species. The clustering of the three new species is similar in MP trees generated from both 28S and 28S + ITS1 datasets where *L. grandis*, new species and *L. liewi*, new species, form sister group with *L. fenestrum* and *L. kederai*, respectively (Fig. 3). *Ligophorus vanbenedenii* DQ157655 and *L. leporinus* from China are not included in the 28S+ITS1 dataset due to the absence of ITS1 sequences in GenBank™. It can also be observed that *Ligophorus* species of *M. buchanani* (*L. liewi*, new species, *L. johorensis*, new species, *L. grandis*, new species, *L. kedahensis*, *L. fenestrum* and *L. kederai*) and *Liza subviridis* (*L. belanaki*, *L. careyensis*, *L. parvicopulatrix*, *L. chelatus* and *L. navjotsodhii*) were clustered in the same subclade within Clade 2 (Fig. 3B). This trend is similar to those found in the MP tree generated from 28S dataset (Fig. 3A).

TAXONOMY

Class Monogenea Carus, 1863

Family Ancyrocephalidae Bychowsky, 1937

Ligophorus liewi, new species

(Figs. 4A, B, 5A–F)

Type host. *Moolgarda buchanani* (Bleeker, 1853).

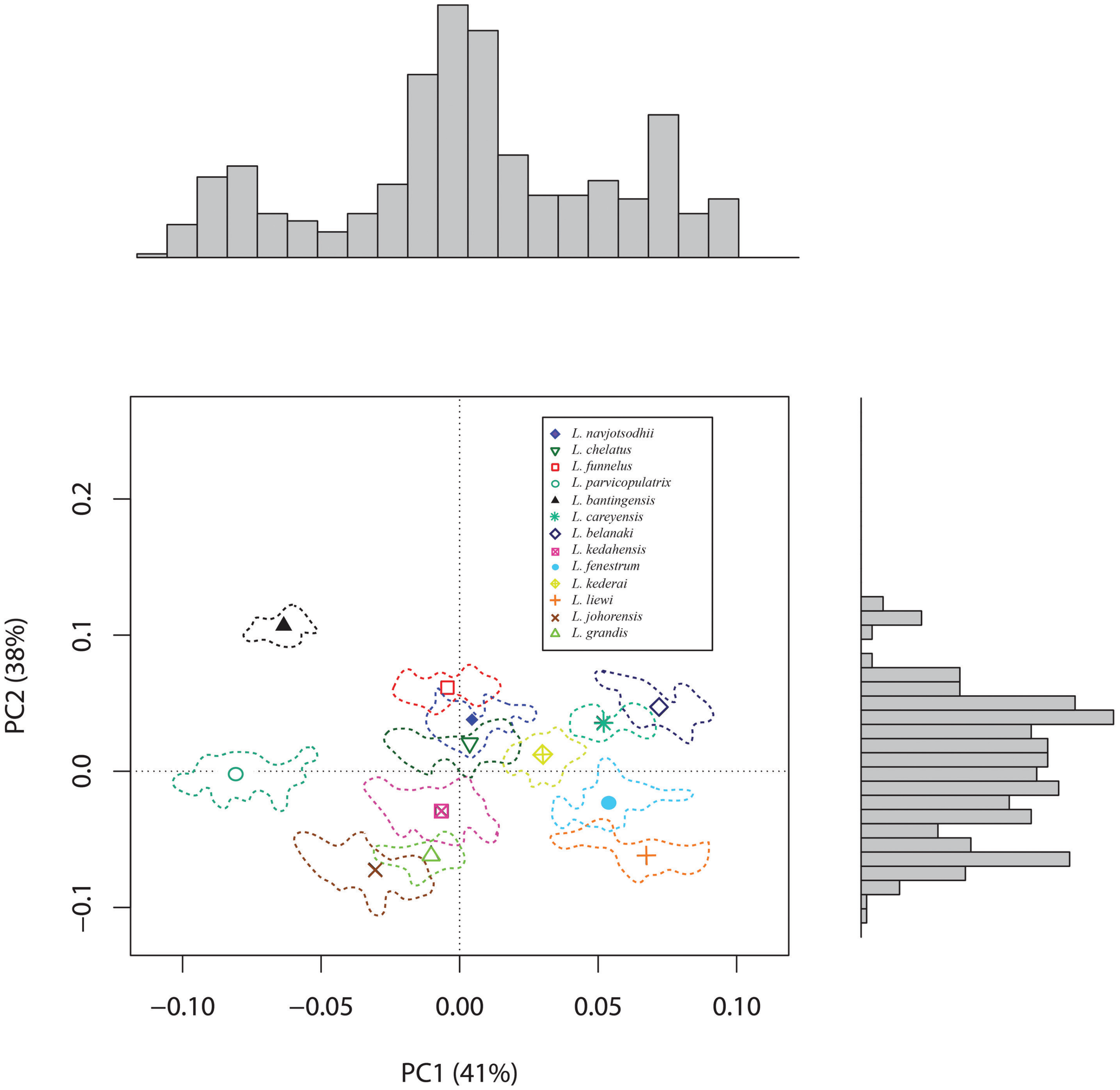


Fig. 1. Principal Component Analysis (PCA) scatterplot of 465 *Ligophorus* specimens based on all hard parts (marginal hook, anchors, bars and male copulatory organ). Vertical and horizontal bar plots indicate one-dimensional summary of the principal component axes, PC1 and PC2.

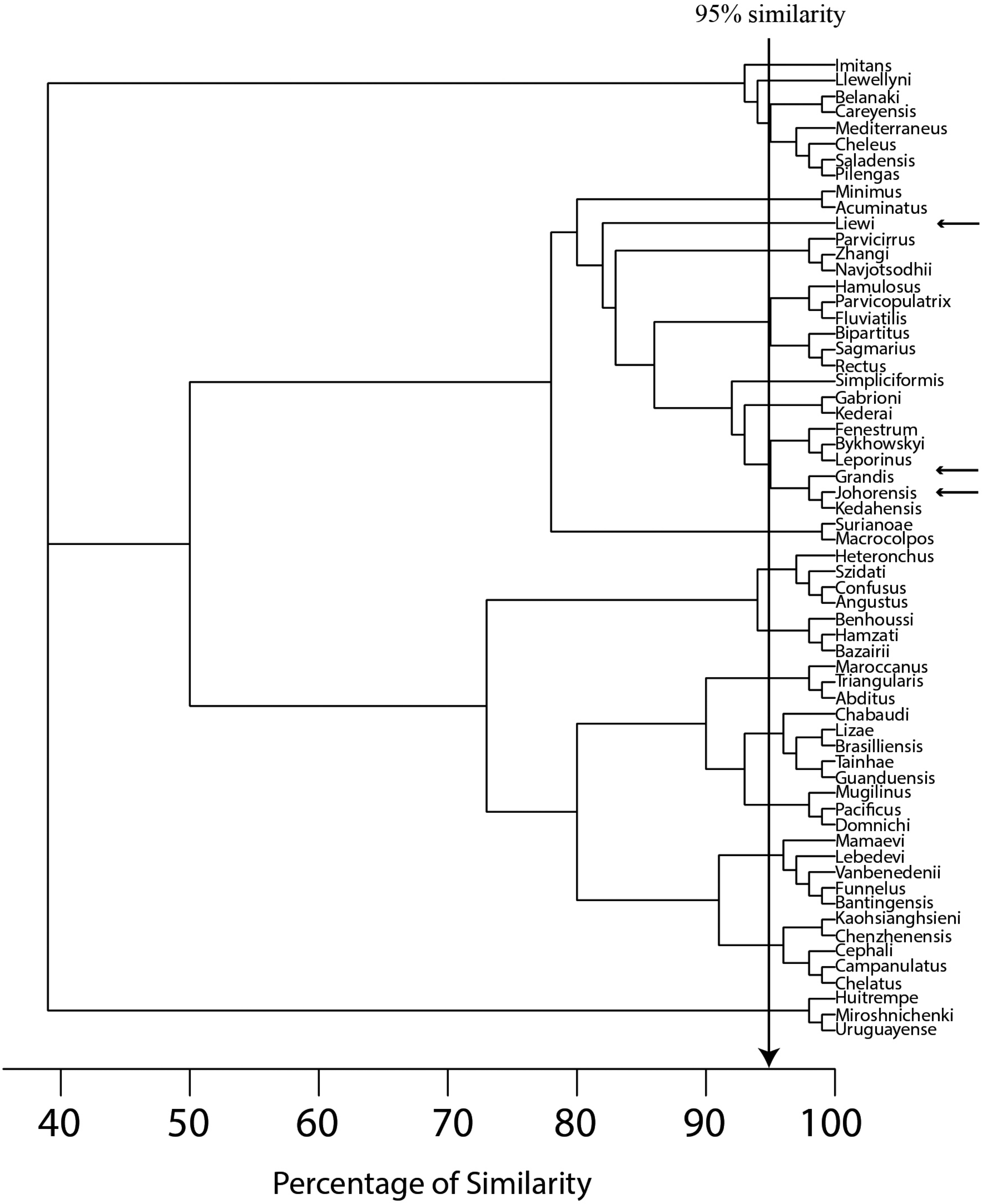
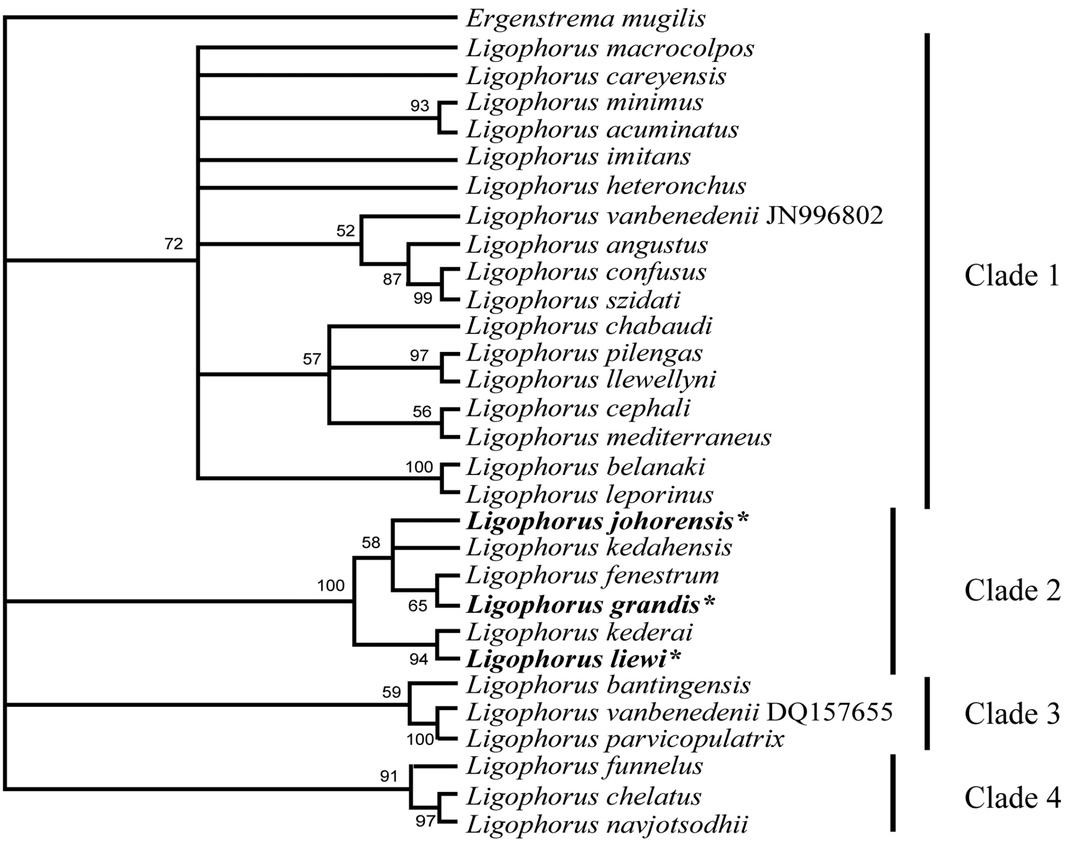


Fig. 2. Dendrogram of 62 *Ligophorus* species based on characteristics of all hard parts (anchors, bars and copulatory organ). The arrows on the far right indicate the positions of the three new species: *L. liewi*, *L. johorensis* and *L. grandis*.

(A) 28S



(B) 28S + ITS1

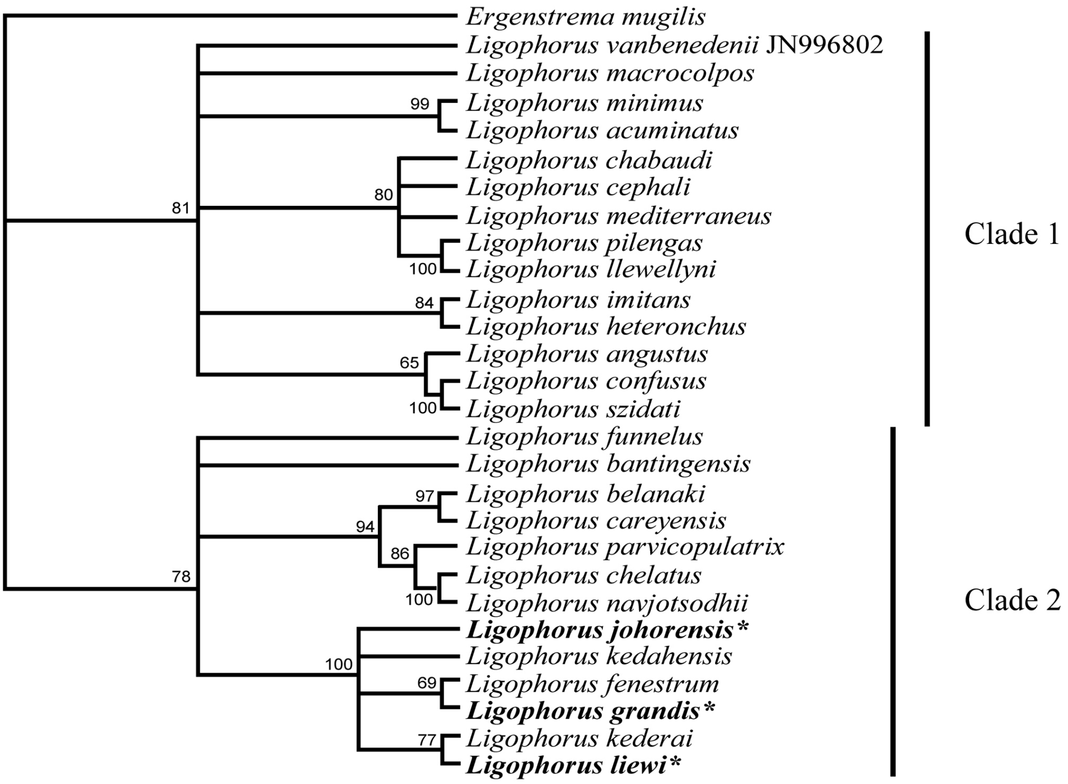


Fig. 3. Maximum-parsimony (MP) trees generated from (A) 28S dataset and (B) 28S+ITS1 dataset using PAUP* with *Ergenstrema mugilis* as the outgroup. Percentages of the bootstrap values are shown along the branches. *denotes new species

Type locality. Straits of Johor, Peninsular Malaysia (01°2'6"N 103°5'4"E).

Materials examined. All specimens collected from the Straits of Johor, Peninsular Malaysia. Holotype: NHMUK 2014.11.10.4, coll. Liew Kim Seng, 19 December 2013. Paratypes: NHMUK 2014.11.10.5–2014.11.10.8, ZRC.PAR.43–ZRC.PAR.44 and MZUM (P) 2014.7(P) – 2014.30(P), coll. Liew Kim Seng, 19 December 2013.

Etymology. The specific name, *liewi* is named for Mr. Liew Kim Seng who collected these monogeneans.

Description. Body elongate, 1064 (823–1270) × 128 (90–166), three pairs of head organs, two pairs pigmented eye spots, posterior with lenses and bigger than anterior pair. Mouth subterminal, ventral. Haptor well demarcated, size 91 (51–144) × 175 (118–214); 14 larval-type marginal hooks, similar, length 12 (10–13); two pairs of anchors; two dorsal anchors, inner length 48 (45–50), outer length 43 (41–45), inner root 19 (18–22), outer root 8 (6–11), point 12 (10–14); two ventral anchors with fenestration, inner length 48 (45–51), outer length 48 (45–49), inner root 18 (12–21), outer root 11 (7–15), point 11 (9–12); two connecting bars: dorsal bar broad V-shape, 48 (54–63) × 6 (5–8); ventral bar inverted V, 48 (44–52) × 9 (6–12), AMP consists of two membranous lateral pieces and a raised median piece, distance between lateral pieces, 8 (6–10). Testis single, elongate, ovoid, postero-dorsal to ovary. Single elongate prostatic reservoir with prostatic duct leaving reservoir to

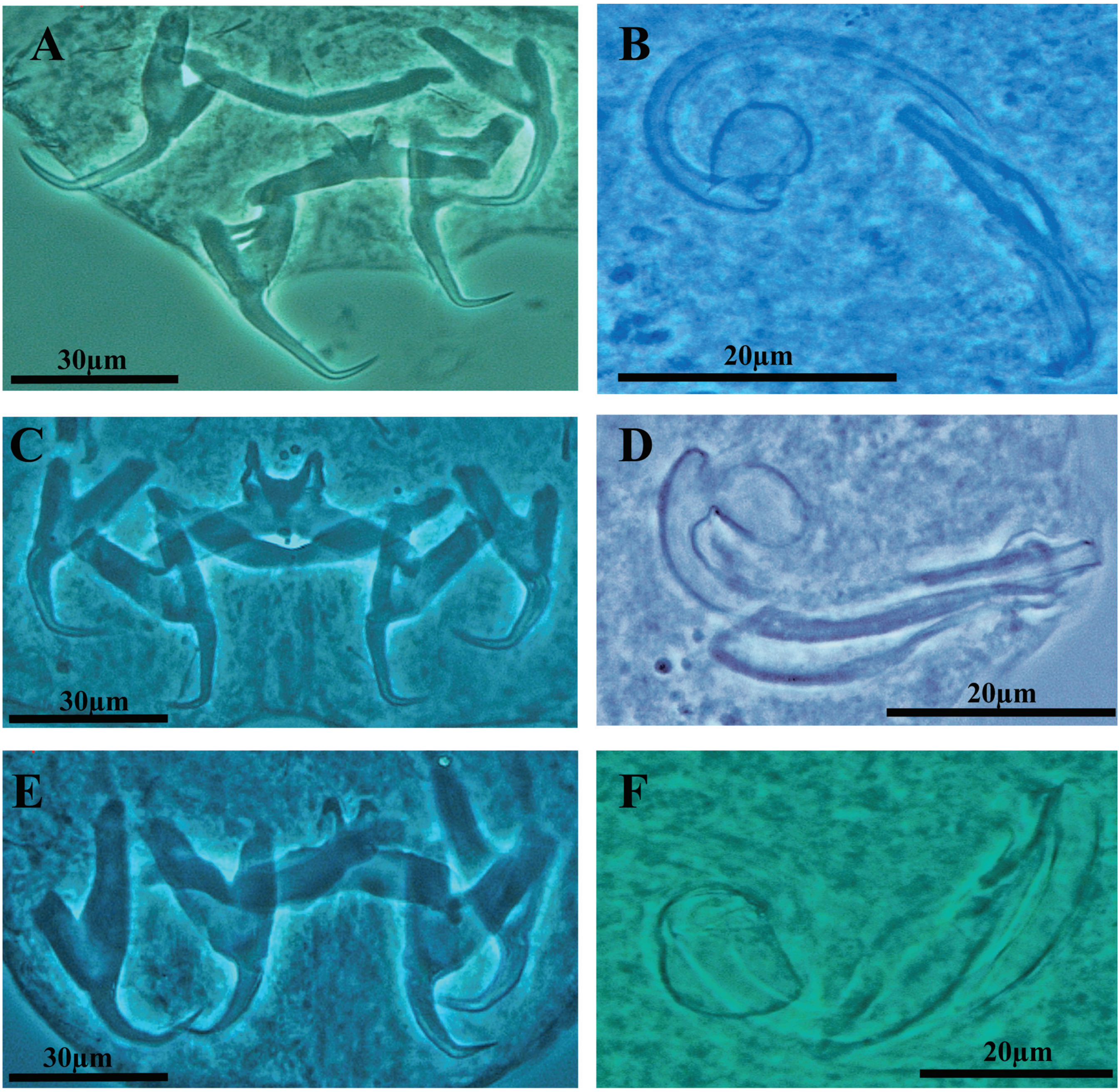


Fig. 4. Photomicrographs of the sclerotised hard parts *Ligophorus liewi*, new species (A & B), *L. johorensis*, new species (C & D) and *L. grandis*, new species (E & F). A, C & E, anchors and bars; B, D & F, male copulatory organ.

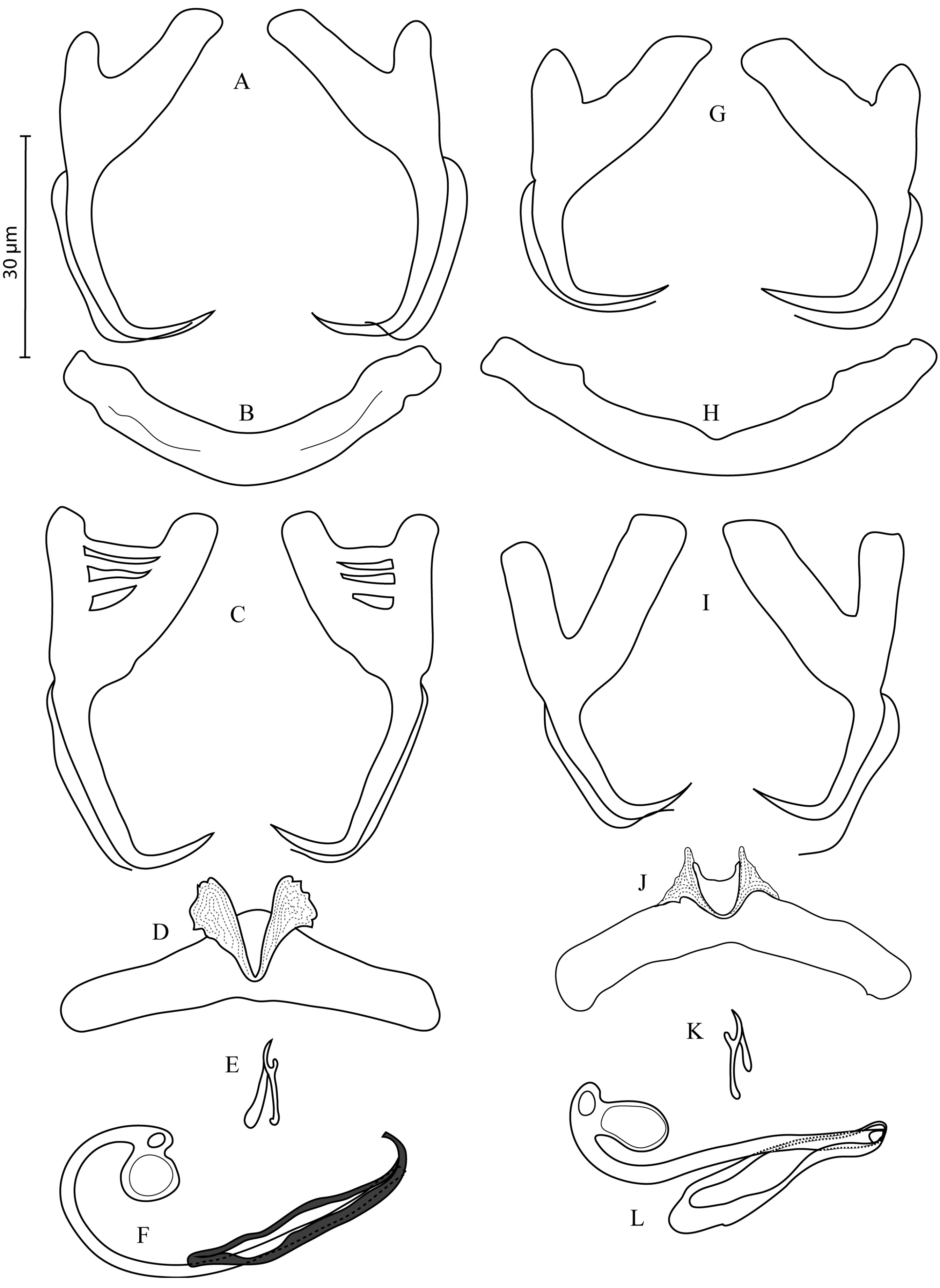


Fig. 5. Sclerotised hard parts of *L. liewi*, new species (A–F) and *L. johorensis*, new species (G–L): A & G, dorsal anchors; B & H, dorsal bar; C & I, ventral anchors; D & J, ventral bar (dorsal view); E & K, marginal hook; F & L, male copulatory organ.

enter bigger lobe of bilobed initial of copulatory tube. Male copulatory organ consists of copulatory tube, length 81 (71–88) with bilobed initial part and a grooved accessory piece with a tapered, bent distal part, 36 (29–42). Ovary J-shaped, elongate with recurved posterior region. Vaginal opening and tube not observed. Vitellarium in lateral fields approximately co-extensive with intestinal caeca, confluent just posterior to intestinal bifurcation, just anterior to ovary and just posterior to cyclocoel.

Differential diagnosis. This is one of three species of the genus *Ligophorus* with fenestrated anchors. *Ligophorus fenestrum* has fenestrated ventral and dorsal anchors (Soo & Lim, 2012) whilst *L. kederai* and *L. liewi*, new species, have only fenestrated ventral anchors (Soo & Lim, 2015). Analyses of molecular data show *L. liewi*, new species, and *L. kederai* are sister taxa (Fig. 3) but *L. liewi*, new species, differs from *L. kederai* in the morphometry of the anchors (Table 1) and morphology of the accessory piece of the copulatory organ; grooved tube with tapered and bent distal end in *L. liewi*, new species, (Fig. 5F) compared to a simple grooved tube in *L. kederai* (Soo & Lim, 2015). *Ligophorus liewi*, new species, is grouped closest to *L. fenestrum* in the PCA scatterplot (Fig. 1) but differs from *L. fenestrum* in the shape and fenestration of the anchors, AMP of the ventral bar and accessory piece of the copulatory organ. *Ligophorus liewi*, new species, has a narrower anchor base, fenestrated ventral anchors, an AMP which comprises of two lateral membranous pieces + a raised median piece and a grooved accessory piece with a tapered and bent distal end (Figs. 5A, C, D, F) compared to a broad anchor base, fenestrated ventral and dorsal anchors, an AMP comprising of a single bifurcated piece and a simple grooved accessory piece in *L. fenestrum* (Soo & Lim, 2012). *Ligophorus liewi*, new species, is also similar to *L. surianoae* in possessing similar anchors, bars and male copulatory organ. However, *L. liewi*, new species, differs in having fenestrated ventral anchors, a shorter dorsal bar 48 (54–63) and a shorter male copulatory tube 81 (71–88) as compared to non-fenestrated anchors, a longer dorsal bar 56 (48–68) and a longer male copulatory tube 99 (95–103) in *L. surianoae*.

Ligophorus johorensis, new species

(Figs. 4C, D, 5G–L)

Type host. *Moolgarda buchanani* (Bleeker, 1853).

Type locality. Straits of Johor, Peninsular Malaysia (01°2'6"N 103°5'4"E).

Materials examined. All specimens collected from the Straits of Johor, Peninsular Malaysia. Holotype: NHMUK 2014.11.10.1, coll. Liew Kim Seng, 19 December 2013. Paratypes: NHMUK 2014.11.10.2–2014.11.10.3, ZRC.PAR.40–ZRC.PAR.42 and MZUM (P) 2014.39(P)–2014.72(P), coll. Liew Kim Seng, 19 December 2013.

Etymology. This species is named after the state of Johor, Peninsular Malaysia.

Description. Body elongate, 1124 (955–1304) × 175 (131–214), three pairs of head organs, two pairs pigmented eye spots, posterior with lenses and bigger than anterior pair. Mouth subterminal, ventral. Haptor well demarcated, size 89 (64–118) × 200 (148–235); 14 larval-type marginal hooks, similar, length 12 (11–13); two pairs of anchors; two dorsal

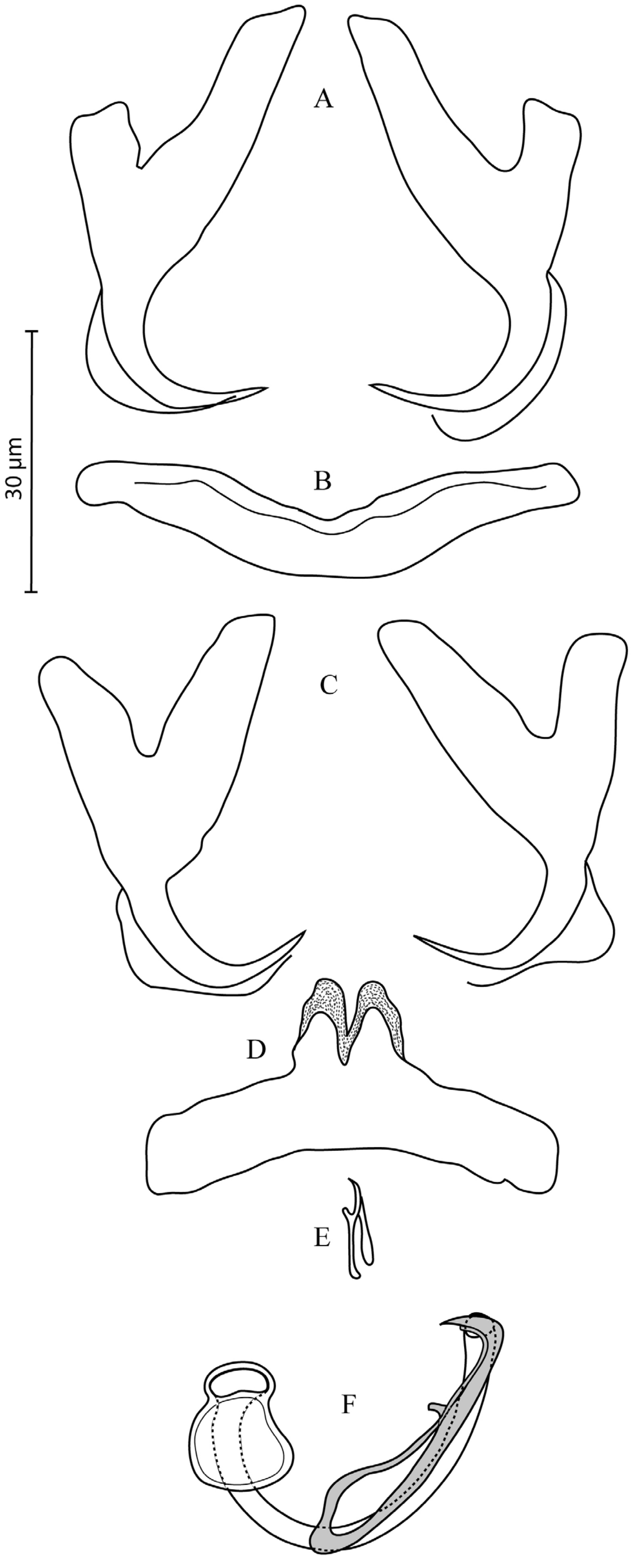


Fig. 6. Sclerotised hard parts of *L. grandis*, new species: A, dorsal anchors; B, dorsal bar; C, ventral anchors; D, ventral bar (dorsal view); E, marginal hook; F, male copulatory organ.

anchors, inner length 38 (35–41), outer length 34 (28–38), inner root 18 (15–21), outer root 9 (5–10), point 12 (10–13); two ventral anchors, inner length 40 (37–43), outer length 39 (35–42), inner root 19 (17–21), outer root 13 (10–14), point 9 (8–10); two connecting bars: dorsal bar broad V, 59 (52–74) × 5 (2–7); ventral bar inverted V, 51 (40–55) × 9 (6–13), AMP consists of two membranous lateral pieces and a raised median piece, distance between lateral pieces, 9 (6–11). Testis single, elongate, ovoid, postero-dorsal to ovary, spermatozoa observed in anterior region. Single elongate prostatic reservoir with prostatic duct leaving reservoir to enter bigger lobe of bilobed initial of copulatory tube. Male copulatory organ consists of copulatory tube, length 53 (46–60) with bilobed initial part and a simple grooved boat-like accessory piece, 30 (25–37). Ovary J-shaped with recurved posterior region; oviduct arises from anterior part of ovary. Vaginal opening and tube not observed. Vitellarium in lateral fields approximately co-extensive with intestinal caeca, confluent just posterior to intestinal bifurcation, just anterior to ovary and just posterior to cyclocoel.

Differential diagnosis. *Ligophorus johorensis*, new species, and *L. kedahensis* which possess anchors and accessory pieces of similar shape (anchors with long blade-like inner roots and a simple grooved boat-like accessory piece) are grouped closest in the NT analysis (Fig. 2). However, *L.*

Table 3. List of *Ligophorus* species used in this study with their host species, locality and GenBank™ accession numbers (*present study)

Monogenean Species	Host Species	Locality	GenBank™ number	
			28S	ITS1
Dactylogyridea Bychowsky 1937				
Ancyrocephalidae Bychowsky, 1937				
Ligophorus navjotsodhii	*Liza subviridis*	Carey Island, Selangor	KM221920*	KM221932*
Ligophorus chelatus	*Liza subviridis*	Carey Island, Selangor	KM221912*	KM221925*
Ligophorus funnelus	*Liza subviridis*	Carey Island, Selangor	KM221914*	KM262662*
Ligophorus parvicopulatrix	*Liza subviridis*	Carey Island, Selangor	KM221921*	KM221933*
Ligophorus bantingensis	*Liza subviridis*	Carey Island, Selangor	KM221909*	KM221922*
Ligophorus careyensis	*Liza subviridis*	Carey Island, Selangor	KM221911*	KM221924*
Ligophorus belanaki	*Liza subviridis*	Carey Island, Selangor	KM221910*	KM221923*
Ligophorus kedahensis	*Moolgarda buchanani*	Langkawi Island, Kedah	KM221917*	KM221929*
Ligophorus fenestrum	*Moolgarda buchanani*	Langkawi Island, Kedah	KM221913*	KM221926*
Ligophorus kederai	*Moolgarda buchanani*	Langkawi Island, Kedah	KM221918*	KM221930*
Ligophorus liewi, new species	*Moolgarda buchanani*	Straits of Johor	KM221919*	KM221931*
Ligophorus johorensis, new species	*Moolgarda buchanani*	Straits of Johor	KM221916*	KM221928*
Ligophorus grandis, new species	*Moolgarda buchanani*	Straits of Johor	KM221915*	KM221927*
Ligophorus acuminatus	*Liza saliens*	Ebro Delta, Spain	JN996816	JN996852
Ligophorus angustus	*Chelon labrosus*	Off Cullera, Spain	JN996804	JN996840
Ligophorus cephali	*Liza cephalus*	Off Cullera, Spain	JN996830	JN996865
Ligophorus chabaudi	*Liza cephalus*	Ebro Delta, Spain	JN996834	JN996869
Ligophorus confusus	*Liza ramada*	Off Cullera, Spain	JN996810	JN996847
Ligophorus heteronchus	*Liza saliens*	Ebro Delta, Spain	JN996812	JN996848
Ligophorus imitans	*Liza ramada*	Ebro Delta, Spain	JN996814	JN996851
Ligophorus leporinus	*Liza cephalus*	Guangdong, China	DQ537380	–
Ligophorus llewellyni	*Liza haematocheila*	Utlyuksky Estuary, Sea of Azov	JN996822	JN996858
Ligophorus macrocolpos	*Liza saliens*	Ebro Delta, Spain	JN996820	JN996857
Ligophorus mediterraneus	*Liza cephalus*	Off Cullera, Spain	JN996828	JN996864
Ligophorus minimus	*Liza saliens*	Ebro Delta, Spain	JN996818	JN996854
Ligophorus pilengas	*Liza haematocheila*	Utlyuksky Estuary, Sea of Azov	JN996826	JN996861
Ligophorus szidati	*Liza aurata*	Ebro Delta, Spain	JN996806	JN996841
Ligophorus vanbenedenii	*Liza aurata*	Ebro Delta, Spain	JN996802	JN996837
Ligophorus vanbenedenii (China)	*Liza cephalus*	Zhangjiang, Guangdong Province, China	DQ157655	–
Outgroup				
Tetraonchidae Bychowsky, 1937				
Ergenstrema mugilis	*Liza ramada*	Ebro Delta, Spain	JN996800	JN996835

johorensis, new species, and *L. kedahensis* differ in the length of the copulatory tube, the width of the dorsal bar and the morphology of the AMP. *Ligophorus johorensis*, new species, has a shorter copulatory tube and wider dorsal bar than *L. kedahensis*; 53 (46–60) and 59 (52–74) in *L. johorensis*, new species, vs. 65 (57–75) and 51 (43–58) in *L. kedahensis*. In terms of the AMP, both species have raised median pieces but in *L. johorensis*, new species, the median piece is raised higher than in *L. kedahensis* and the distance between its membranous lateral pieces is shorter; 9 (6–11) in *L. johorensis*, new species, (Fig. 5J) and 12 (7–15) in *L. kedahensis* (Soo & Lim, 2012). *Ligophorus johorensis*, new species, is grouped closest to *L. grandis*, new species, in the PCA scatterplot (Fig. 1) but can be differentiated from *L. grandis*, new species, in the morphology of the AMP; two lateral pieces and a raised median piece in *L. johorensis*, new species, (Fig. 5J) compared to a single bifurcating piece in *L. grandis*, new species (Fig. 6D). *Ligophorus johorensis*, new species, also closely resembles *L. zhangi* Dmitrieva, Gerasev, Gibson, Pronkina and Galli, 2012 in the general shape of its anchors, bars and male copulatory organ but it differs in the shape of the AMP of the ventral bar and length of the dorsal bar. *Ligophorus johorensis*, new species, has a raised median piece and a longer dorsal bar, 59 (52–74) compared to a flat median piece and shorter dorsal bar, 50µm and 52µm from two specimens of *L. zhangi*. *Ligophorus johorensis*, new species, is similar to *L. simpliciformis* in possessing morphologically similar ventral bars. However, *L. johorensis*, new species, differs from *L. simpliciformis* in having a longer male copulatory organ and accessory piece: 53 (46–60) and 30 (25–37) in *L. johorensis*, new species, vs. 43 (40–45) and 22 (20–23) in *L. simpliciformis.*

Ligophorus grandis, new species

(Figs. 4E, F, 6A–F)

Type host. *Moolgarda buchanani* (Bleeker, 1853).

Type locality. Straits of Johor, Peninsular Malaysia (01°2′6″N 103°5′4″E)

Materials examined. All specimens collected from the Straits of Johor, Peninsular Malaysia. Holotype: NHMUK 2014.11.10.9, coll. Liew Kim Seng, 19 December 2013. Paratypes: NHMUK 2014.11.10.10–2014.11.12, ZRC.PAR.45–ZRC.PAR.47 and MZUM (P) 2014.108(P)–2014.122(P), coll. Liew Kim Seng, 19 December 2013.

Etymology. This species is named after its size, being the largest Malaysian *Ligophorus* species.

Description. Body elongate, 1966 (1355–2167) × 321 (200–379), three pairs of head organs, two pairs pigmented eye spots, posterior with lenses and bigger than anterior pair. Mouth subterminal, ventral. Haptor well demarcated, size 96 (74–105) × 145 (123–159); 14 larval-type marginal hooks, similar, length 12 (10–13); two pairs of anchors; two dorsal anchors, inner length 44 (42–45), outer length 35 (34–37), inner root 21 (18–23), outer root 10 (7–12), point 11 (10–13); two ventral anchors, inner length 41 (39–43), outer length 39 (37–42), inner root 20 (18–22), outer root 12 (8–14), point 10 (8–10); two connecting bars: dorsal bar broad V, 54 (51–57) × 8 (6–9); ventral bar inverted V, 45 (43–49) × 12 (9–14), AMP consists of single bifurcated piece, distance between bifurcations, 3 (1–4). Testis single, elongate, ovoid, comparatively large, postero-dorsal to ovary, spermatozoa observed in anterior region. Single prostatic reservoir with prostatic duct leaving reservoir to enter bigger lobe of bilobed initial of copulatory tube. Male copulatory organ consists of copulatory tube, length 59 (52–63) with bilobed initial part and a grooved accessory piece with an expanded hook-shaped distal end 34 (31–40). Ovary J-shaped, large, elongate with recurved posterior region. Vaginal opening and tube not observed. Vitellarium in lateral fields approximately co-extensive with intestinal caeca, confluent just posterior to intestinal bifurcation, just anterior to ovary and just posterior to cyclocoel.

Differential diagnosis. *Ligophorus grandis*, new species, is the largest *Ligophorus* species collected from Malaysian waters (Table 1). *Ligophorus grandis*, new species, is most similar to *L. johorensis*, new species, and *L. kedahensis* based on NT analysis (Fig. 2) but it can be differentiated from *L. kedahensis* and *L. johorensis*, new species, in the morphology of its AMP and accessory piece of the copulatory organ. *Ligophorus grandis*, new species, possesses a single bifurcating AMP (Fig. 6D), compared to a raised median piece with membranous lateral pieces in both *L. kedahensis* (Soo & Lim, 2012) and *L. johorensis*, new species (Fig. 5J). In terms of the morphology of the accessory piece, *L. grandis*, new species, has a grooved accessory piece with an expanded hook-shaped distal end (Fig. 6F), compared to a simple grooved boat-like accessory piece in both *L. kedahensis* (Soo & Lim, 2012) and *L. johorensis*, new species (Fig. 5L). *Ligophorus grandis*, new species, also differs from *L. fenestrum* in having non-fenestrated anchors (Figs. 6A, C) despite forming sister group with *L. fenestrum* in the MP trees (Fig. 3). *Ligophorus grandis*, new species, is most similar to *L. bykhowskyi* in possessing similar anchors, dorsal bar and male accessory piece. However, *L. grandis*, new species, differs in possessing a single bifurcating AMP, a shorter ventral bar 45 (43–49), longer dorsal anchor points 11 (10–13) and longer ventral anchor points 10 (8–10) as compared to an AMP consisting of two membranous lateral pieces + a raised median piece, a longer ventral bar 49 (45–52), shorter dorsal anchor points 10 (9–10) and shorter ventral anchor points 7 (6–7) in *L. bykhowskyi*. It is also observed that *L. grandis*, new species, has a morphologically similar ventral bar to *L. bipartitus* but differs in having larger anchors, a longer male copulatory tube 59 (52-63) and longer accessory piece 34 (31–40) compared to smaller anchors, a shorter male copulatory tube 42 (38–45) and shorter accessory piece 24 (23–26) in *L. bipartitus*.

DISCUSSION

The description of these three new species increases the number of *Ligophorus* species recorded from Malaysian waters to a total of 13, and a global total of 62 species. Soo & Lim (2012, 2015) described three *Ligophorus* species (*L.*

Table 4. Similarities (as sequence identity) of partial 28S rDNA sequences (D1–D2 domain) from *Ligophorus* species. Abbreviation of species given in the Materials and Methods. (*Sequences from new species in this study; ** Sequences of *L. vanbenedenii* DQ157655 and *L. parvicopulatrix*).

	Min	Acu	Imi	Het	Con	Szi	Ang	Van	Mac	Pil	Lle	Cha	Cep	Med
Min	1.000	0.967	0.951	0.942	0.918	0.921	0.918	0.932	0.946	0.935	0.935	0.938	0.943	0.937
Acu	—	1.000	0.956	0.948	0.930	0.934	0.932	0.942	0.956	0.950	0.950	0.945	0.953	0.948
Imi	—	—	1.000	0.964	0.927	0.927	0.922	0.940	0.958	0.946	0.946	0.953	0.950	0.946
Het	—	—	—	1.000	0.929	0.932	0.922	0.950	0.951	0.950	0.950	0.956	0.953	0.942
Con	—	—	—	—	1.000	0.987	0.943	0.934	0.924	0.929	0.927	0.934	0.934	0.921
Szi	—	—	—	—	—	1.000	0.950	0.940	0.930	0.932	0.930	0.937	0.940	0.927
Ang	—	—	—	—	—	—	1.000	0.932	0.922	0.929	0.927	0.934	0.932	0.922
Van	—	—	—	—	—	—	—	1.000	0.946	0.945	0.945	0.948	0.951	0.940
Mac	—	—	—	—	—	—	—	—	1.000	0.958	0.958	0.958	0.969	0.961
Pil	—	—	—	—	—	—	—	—	—	1.000	0.996	0.975	0.979	0.967
Lle	—	—	—	—	—	—	—	—	—	—	1.000	0.975	0.982	0.967
Cha	—	—	—	—	—	—	—	—	—	—	—	1.000	0.979	0.961
Cep	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.975
Med	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000
VanC**	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Par**	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Bel	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Car	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Che	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Nav	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fun	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fen	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gra*	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Joh*	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Keda	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Kede	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lie*	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ban	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lep	—	—	—	—	—	—	—	—	—	—	—	—	—	—

VanC**	Par**	Bel	Car	Che	Nav	Fun	Fen	Gra*	Joh*	Keda	Kede	Lie*	Ban	Lep
0.900	0.900	0.921	0.913	0.892	0.906	0.879	0.818	0.794	0.808	0.836	0.823	0.805	0.884	0.916
0.898	0.898	0.930	0.922	0.895	0.906	0.890	0.831	0.805	0.818	0.842	0.837	0.816	0.885	0.927
0.898	0.898	0.927	0.927	0.909	0.926	0.900	0.829	0.821	0.827	0.852	0.836	0.819	0.893	0.922
0.887	0.887	0.929	0.927	0.898	0.909	0.884	0.827	0.815	0.816	0.852	0.829	0.808	0.882	0.926
0.890	0.890	0.914	0.908	0.900	0.898	0.884	0.815	0.808	0.807	0.824	0.829	0.811	0.882	0.908
0.897	0.897	0.918	0.909	0.900	0.895	0.887	0.821	0.808	0.807	0.829	0.827	0.810	0.889	0.911
0.884	0.884	0.913	0.901	0.884	0.882	0.866	0.818	0.810	0.816	0.831	0.832	0.813	0.884	0.908
0.889	0.889	0.924	0.909	0.890	0.895	0.871	0.824	0.810	0.810	0.844	0.832	0.810	0.884	0.921
0.893	0.893	0.943	0.940	0.900	0.911	0.893	0.823	0.802	0.818	0.834	0.823	0.811	0.892	0.940
0.887	0.887	0.953	0.927	0.905	0.909	0.884	0.823	0.810	0.824	0.842	0.834	0.815	0.882	0.951
0.887	0.887	0.954	0.929	0.903	0.908	0.882	0.823	0.810	0.824	0.842	0.837	0.815	0.882	0.953
0.890	0.890	0.950	0.927	0.906	0.913	0.895	0.826	0.811	0.824	0.844	0.831	0.815	0.890	0.946
0.892	0.892	0.954	0.930	0.901	0.909	0.887	0.821	0.803	0.818	0.842	0.834	0.813	0.890	0.951
0.893	0.893	0.937	0.921	0.895	0.903	0.877	0.816	0.805	0.815	0.840	0.834	0.811	0.892	0.934
1.000	**1.000**	0.879	0.874	0.890	0.892	0.872	0.810	0.782	0.799	0.813	0.810	0.808	0.895	0.879
—	1.000	0.879	0.874	0.890	0.892	0.872	0.810	0.782	0.799	0.813	0.810	0.808	0.895	0.879
—	—	1.000	0.924	0.889	0.893	0.869	0.815	0.803	0.813	0.823	0.823	0.803	0.868	0.990
—	—	—	1.000	0.884	0.890	0.872	0.818	0.810	0.818	0.826	0.831	0.808	0.874	0.919
—	—	—	—	1.000	0.972	0.926	0.810	0.794	0.815	0.815	0.815	0.805	0.882	0.887
—	—	—	—	—	1.000	0.922	0.813	0.792	0.815	0.821	0.823	0.815	0.884	0.892
—	—	—	—	—	—	1.000	0.815	0.799	0.815	0.821	0.811	0.797	0.884	0.868
—	—	—	—	—	—	—	1.000	0.900	0.882	0.889	0.882	0.868	0.792	0.818
—	—	—	—	—	—	—	—	1.000	0.895	0.897	0.879	0.863	0.784	0.805
—	—	—	—	—	—	—	—	—	1.000	0.895	0.882	0.879	0.795	0.819
—	—	—	—	—	—	—	—	—	—	1.000	0.866	0.856	0.811	0.824
—	—	—	—	—	—	—	—	—	—	—	1.000	0.905	0.805	0.827
—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.781	0.808
—	—	—	—	—	—	—	—	—	—	—	—	—	1.000	0.868
—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.000

kedahensis, *L. fenestrum* and *L. kederai*) in *M. buchanani* from Langkawi Island but the present three new species were not found there.

Blasco-Costa et al. (2012) noted that *L. vanbenedenii* DQ157655 (from China) is different from *L. vanbenedenii* JN996802 (from the type-locality, Mediterranean Sea) and suggested that *L. vanbenedenii* DQ157655 might belong to a different genus based on its high divergence from the other *Ligophorus* species. Partial 28S rDNA sequence of *L. vanbenedenii* DQ157655 is 88.9% similar to *L. vanbenedenii* JN996802 but is 100% similar to *L. parvicopulatrix* from *Liza subviridis* from Malaysia (Table 4), suggesting that *L. vanbenedenii* DQ157655 is in fact *L. parvicopulatrix* (Lim et al., 2010). *Ligophorus vanbenedenii* DQ157655 was collected from *Liza cephalus* in Zhanjiang, Guangdong Province, China (Wu et al., 2006). It is highly possible that the host species of *L. vanbenedenii* DQ157655 could have been misidentified as *L. cephalus*, as mullets are shown to have very similar morphological characters (Durand et al., 2012).

Ligophorus leporinus was reassigned from the genus *Ancyrocephalus* to the genus *Ligophorus* by Gusev (1985) because of its presence in a mullet host (*L. cephalus*). Zhang and Ji (1981) reaffirmed the assignment of this species based on the description of its sclerotised hard parts. In a recent study, Sarabeev et al. (2013) claimed that *L. leporinus* is morphologically similar to *Kriboetrema rectangulus* Sarabeev, Rubtsova, Yang & Balbuena, 2013 in possessing similar hard parts (anchors and bars of similar shape, coiled copulatory organ, simple concave accessory piece of the copulatory organ) and concluded it as 'incertae sedis'. However, the 28S rDNA sequence data showed *L. leporinus* is 99% similar to *L. belanaki* (Table 4) and forms a sister group with *L. belanaki* in the MP tree inferred from the 28S dataset (Fig. 3A). A previous study evaluating the interspecific relationship among *Ligophorus* species by Blasco-Costa et al. (2012) also found that *L. leporinus* is grouped with *L. mediterraneus*, *L. llewellyni*, *L. pilengas*, *L. cephali* and *L. chabaudi* based on the analysis of the 28S rDNA region. Therefore, our present study and that of Blasco-Costa et al. (2012) indicate that *L. leporinus* is most probably a valid *Ligophorus* species. However, molecular data from other parts of the genome are needed to confirm or refute this.

The MP trees presented in this study show that *L. liewi*, new species, *L. johorensis*, new species and *L. grandis*, new species, are grouped with *L. kederai*, *L. fenestrum* and *L. kedahensis* found in the same host species, *M. buchanani* (Fig. 3). The same can be observed for *L. chelatus*, *L. navjotsodhii*, *L. belanaki*, *L. parvicopulatrix* and *L. careyensis* from *Liza subviridis* (Fig. 3B). These results indicate *Ligophorus* species from the same host are more closely related and suggest possible intra-host speciation. This trend has also been reported by Blasco-Costa et al. (2012) for *Ligophorus* species from the Mediterranean and Black seas. Thus, the present study provides further evidence that intra-host speciation is probably one of the more important ecological processes involved in species diversification of the ectoparasitic genus *Ligophorus*.

ACKNOWLEDGEMENTS

The authors would like to thank Peter KL Ng and Tommy Tan (National University of Singapore) for their help in obtaining host specimens and to Liew Kim Seng for collecting the fish and monogenean specimens. This study was funded by two UMRG grants (RP004D-13SUS and RP008-2012B). This paper forms part of the first author's PhD thesis.

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