Taxonomy and molecular phylogeny of the Asian *Paraleucophenga* Hendel (Diptera, Drosophilidae)

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This paper reports on nine Asian species of the genus *Paraleucophenga*, of which four are new to science: *Paraleucophenga brevipennis* sp. nov., *Paraleucophenga hirtipennis* sp. nov., *Paraleucophenga longiseta* sp. nov., and *Paraleucophenga tanydactylia* sp. nov. We also report on a new synonym, *Paraleucophenga shanyinensis* Chen & Toda, 1994 syn. nov. A key to all of the species examined, based on morphological data, is provided, together with a 'molecular' key to seven *Paraleucophenga* species based on DNA sequence data of the mitochondrial *NADH dehydrogenase subunit 2* (*ND2*) gene. The phylogenetic relationships among seven *Paraleucophenga* species are reconstructed based on DNA sequences of the mitochondrial *ND2* gene, using two *Leucophenga* species as outgroups. © 2009 The Linnean Society of London, *Zoological Journal of the Linnean Society*, 2009, 155, 615–629.

ADDITIONAL KEYWORDS: East Asia – mtDNA – new species – new synonym – Steganinae.

INTRODUCTION

The genus *Paraleucophenga* Hendel, 1914 of the subfamily Steganinae was known from one Afrotropical and seven Oriental species (Walker, 1857; Okada, 1956, 1988; Bächli, 1971; Singh & Negi, 1992; Chen & Toda, 1994; Sidorenko, 1998). Adults of *Paraleucophenga* are slender, but are relatively large in body size (i.e., body length) (~4–5 mm). The adults surround humans at the forest edge, where they can also be collected from tree trunks, but are very difficult to detect in dim forest. In comparison with adults of *Paraleucophenga*, adults of the genera *Amiota* Loew, 1982 and *Phortica* Schiner, 1862 seldom appear around humans at the forest edge, but pursue humans in the forest.

Okada (1989) assigned the genus *Paraleucophenga* in the tribe Leucophengini, along with the genus *Leucophenga* Mik, 1886, based on a phenetic analysis with 14 adult morphological characters. Whereas Grimaldi (1990), based on a cladistic analysis of 217

adult characters, assigned *Paraleucophenga* in the tribe Gitonini, along with some other genera (e.g. *Amiota*, *Cacoxenus* Loew, 1858, and *Gitona* Meigen, 1830). Morphological similarity between *Paraleucophenga* and *Leucophenga*, such as the silvery pollinosity on male scutum and abdominal tergites (Figs 1–8), supports Okada's classification; whereas the behavioral similarity between *Paraleucophenga* and *Amiota* favour Grimaldi's proposal.

Lin & Wheeler (1972) described the abdominal colour pattern of *Paraleucophenga invicta* (Walker, 1857) in a female specimen. However, this character is usually much less variable in females than in males among species. In the present study we taxonomically surveyed ten species of *Paraleucophenga* from East and South East Asia, four of which were found in southern China and are described as new species; and found that the fifth sternites of males vary (Figs 9–15). Phylogenetic relationships among eight (as one species is present as a junior synonym of another, there are actually seven) of these species are investigated based on DNA sequences of the mitochondrial *NADH dehydrogenase subunit 2* (*ND2*) gene, using two *Leucophenga* species as outgroups.

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MATERIAL AND METHODS

TAXON SAMPLING

The examined specimens are deposited in the following institutions: Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ); Department of Zoology, National Science Museum, Tokyo, Japan (NSMT); Department of Entomology, South China Agricultural University (SCAU); and Systematic Entomology, the Hokkaido University Museum, Hokkaido University, Sapporo, Japan (SEHU).

For taxonomical terminology and indices, see Chen & Toda (2001). New records are indicated by an asterisk (*).

SAMPLES FOR MOLECULAR PHYLOGENETIC STUDY

Eight *Paraleucophenga* species, each represented by between one and five populations, were sampled for phylogenetic analyses. Two *Leucophenga* species, each represented by a single population, were used as outgroups. The collection data for all of these samples are shown in Table 1.

DNA EXTRACTION, PCR, AND SEQUENCING

Samples were preserved in 70% ethanol immediately after collection. Total DNA was extracted from part of a single fly using the standard phenol/chloroform method (Sambrook, Fritsch & Maniatis, 1989), with a slight modification. The primers used to amplify the

ND2 gene were 5'-AAGCTACTGGGTTTCATACC-3' (forward; Park, 1999) and 5'-ATATTTACAGCTTTGA AGG-3' (reverse; designed specifically for the present study). The PCR reaction mixture (25 µL) contains 0.4 µmol of each primer, 0.2 mmol of each deoxyribonucleoside triphosphate (dNTP), 2.5 mmol MgCl₂, and 0.5 U of *Taq* polymerase. Each of the 35 thermocycles included 1 min denaturing at 94 °C, 1 min annealing at 54 °C, and 1 min elongation at 72 °C. The purified PCR product was then subjected to a sequencing reaction. For a few samples, the purified products were cloned into pMD20-T vector, and were then transformed into *Escherichia coli* TG-1 as a host. Then some positive clones were selected, and one of them was used as the template for sequencing reactions. The sequences were determined using an ABI 3730 automatic sequencer. Accession numbers for all of the newly determined sequences are listed in Table 1.

TEST OF NUCLEOTIDE SUBSTITUTION SATURATION

Substitution saturation resulting from multiple changes at the same site can overwhelm the phylogenetic signal, and can thus effect the phylogenetic reconstruction. It was shown that phylogenetic information is essentially lost when the observed index of substitution saturation (*I*_{ss}) in sequences exceeds the critical index of substitution saturation (*I*_{ss}. *c*)

Table 1. Collection data of samples, and accession numbers of the sequences newly determined in this study

Samples	Collection site	Latitude and longitude	Accession No.
<i>Leucophenga angusta</i>	Tokyo, Japan	35°36'N, 139°23'E	EU180490
<i>Leucophenga quadripunctata</i>	Tokyo, Japan	35°36'N, 139°23'E	EU180491
<i>Paraleucophenga argentosa</i> -YJ	Yamanashi, Japan	35°39'N, 139°07'E	EU180492
<i>Paraleucophenga argentosa</i> -KJ	Kumamoto, Japan	32°37'N, 130°51'E	EU180493
<i>Paraleucophenga shanyinensis</i>	Huajiang, Guangxi, China	25°51'N, 110°27'E	EU180494
<i>Paraleucophenga emeiensis</i> -JX	Guanshan, Jiangxi, China	28°30'N, 114°31'E	EU180495
<i>Paraleucophenga emeiensis</i> -YN	Xishuangbanna, Yunnan, China	21°41'N, 101°25'E	EU180496
<i>Paraleucophenga emeiensis</i> -SC	Fengtongzhai, Sichuan, China	30°34'N, 102°53'E	EU180497
<i>Paraleucophenga emeiensis</i> -FJ	Wuyishan, Fujian, China	27°43'N, 117°57'E	EU180498
<i>Paraleucophenga javana</i> -YN	Xishuangbanna, Yunnan, China	21°41'N, 101°25'E	EU180499
<i>Paraleucophenga javana</i> -GX	Nonggang, Guangxi, China	22°22'N, 106°51'E	EU180500
<i>Paraleucophenga javana</i> -GD	Zhaoqing, Guangdong, China	23°10'N, 112°34'E	EU180501
<i>Paraleucophenga tanydactylia</i> sp. nov.	Xishuangbanna, Yunnan, China	22°20'N, 100°51'E	EU180507
<i>Paraleucophenga brevipenis</i> sp. nov. -GD1.	Nanling, Guangdong, China	25°00'N, 113°24'E	EU180502
<i>Paraleucophenga brevipenis</i> sp. nov. -GD2	Nanling, Guangdong, China	25°00'N, 113°24'E	EU180503
<i>Paraleucophenga hirtipenis</i> sp. nov.	Shibilianshan, Yunnan, China	25°12'N, 104°35'E	EU180506
<i>Paraleucophenga longiseta</i> sp. nov. -JX	Guanshan, Jiangxi, China	28°30'N, 114°31'E	EU180504
<i>Paraleucophenga longiseta</i> sp. nov. -GX	Maershan, Guangxi, China	25°51'N, 110°27'E	EU180505

Table 2. Uncorrected pairwise p-distances among the *ND2* sequences of the *Paraleucophenga* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 <i>P. shanyinensis</i>															
2 <i>P. argentosa</i> -YJ	0.018														
3 <i>P. argentosa</i> -KJ	0.018	0.000													
4 <i>P. brevipenis</i> sp. nov.-GD1	0.107	0.105	0.105												
5 <i>P. brevipenis</i> sp. nov.-GD2	0.100	0.098	0.098	0.007											
6 <i>P. emeiensis</i> -FJ	0.170	0.168	0.168	0.182	0.176										
7 <i>P. emeiensis</i> -JX	0.167	0.168	0.168	0.182	0.176	0.018									
8 <i>P. emeiensis</i> -SC	0.168	0.170	0.170	0.177	0.171	0.016	0.014								
9 <i>P. emeiensis</i> -YN	0.165	0.167	0.167	0.180	0.174	0.018	0.016	0.010							
10 <i>P. hirtipenis</i> sp. nov.	0.090	0.094	0.094	0.044	0.039	0.172	0.170	0.169	0.164						
11 <i>P. javana</i> -GD	0.124	0.128	0.128	0.136	0.131	0.172	0.173	0.172	0.172	0.126					
12 <i>P. javana</i> -GX	0.122	0.126	0.126	0.133	0.128	0.170	0.171	0.170	0.170	0.123	0.006				
13 <i>P. javana</i> -YN	0.125	0.129	0.129	0.135	0.130	0.172	0.173	0.172	0.172	0.127	0.009	0.009			
14 <i>P. longiseta</i> sp. nov.-GX	0.089	0.086	0.086	0.105	0.100	0.173	0.174	0.171	0.172	0.097	0.130	0.130	0.131		
15 <i>P. longiseta</i> sp. nov.-JX	0.089	0.086	0.086	0.105	0.100	0.173	0.174	0.171	0.172	0.097	0.130	0.130	0.131	0.000	
16 <i>P. tanydactylia</i> sp. nov.	0.135	0.136	0.136	0.143	0.140	0.188	0.193	0.194	0.194	0.138	0.055	0.051	0.058	0.142	0.142

The numbers in bold show the intraspecific distances.

(Xia *et al.*, 2003). We used the program DAMBE4.5.32 to test whether *I*_{ss} was lower than *I*_{ss}. *c*.

DIAGNOSING *PARALEUCOPHENG*A SPECIES USING DNA SEQUENCES

All the *ND2* sequences, including those of the outgroup taxa, were aligned using the ClustalW (Thompson, Higgins & Gibson, 1994) method, and were then modified manually. The resulting alignment was used for diagnostic character (nucleotide position) selection, the DNA substitution model test, as well for phylogenetic reconstruction. Groups of nucleotide positions were selected as characters to diagnose different taxa (pairs of species or species clusters) in the genus *Paraleucophenga*. For this, less degenerated positions showing amino acid replacement between (but not within) the two taxa compared were preferentially considered. A 'molecular' key using the selected positions as diagnostic characters was elaborated to distinguish between the *Paraleucophenga* species for which *ND2* sequences were available.

PHYLOGENETIC ANALYSIS

A DNA substitution model was selected using Modeltest 3.7 (Posada & Crandall, 1998), and phylogenetic analyses were performed with the maximum parsimony (MP) and maximum likelihood (ML) methods using PAUP*4.0b10 (Swofford, 2003), and with the Bayesian inference (BI) method using MrBayes 3.1 (Ronquist & Huelsenbeck, 2003). Sites with gaps were treated as missing data in the MP analysis, but were completely deleted in the ML and BI analyses. Calculation of basic statistical quantities of DNA sequences were implemented in MEGA 3.1 (Kumar, Tamura & Nei, 2004). The heuristic search of MP trees was performed with initial trees obtained by 100 replicates of random addition, and branch swapping using the *tree* bisection and reconnection (TBR) algorithm. The K81uf + G (two transversion parameters, model 1, unequal frequencies, and a gamma-distributed substitution rate across sites) model was selected for the ML analysis, with parameters assigned as follows: base frequencies of A, C, G, and T are 0.3695, 0.0974, 0.0584, and 0.4746, respectively; substitution rates for the classes A–C, A–G, A–T, C–G, C–T, and G–T were 1.0000, 12.7822, 1.9286, 1.9286, 12.7822, and 1.0000, respectively; the proportion of invariable sites = 0; gamma distribution shape parameter = 0.2820. For the Bayesian analysis with MrBayes 3.1 (Ronquist & Huelsenbeck, 2003), a site-specific rate model was used, allowing the first codon position to have six

substitution types, whereas the second and third codon positions were only allowed to have two substitution types. For all of the codon positions, a gamma-distributed rate was assumed. For the MP and ML analyses, the bootstrap percentages (BPs; 1000 replicates) were calculated to evaluate the node confidences.

TAXONOMIC ACCOUNT

*PARALEUCOPHENG*A HENDEL, 1914

Paraleucophenga Hendel, 1914: 114.

Type species: Helomyza invicta Walker, 1857: 130.

Diagnosis: Modified from Bächli (1971) and Okada (1988). Frons narrow, less than one third of head width; arista with long dorsal and fine ventral branches; scutellum usually with small setae along margin; katapisternal setae 3; abdominal tergites with silvery white pollinosity; cercus elongated below and slightly constricted submedially (Figs 16, 18, 20, 22); paramere with tooth comb (Figs 17, 19, 21, 23); aedeagal apodeme sub-basally narrow, arched, distally broadened (Figs 17, 19, 21, 23).

Description: Body slender, usually longer than 4.0 mm.

Head: Eye dark red. Ocellar triangle: black, with one pair of small setae above ocellar setae. Frons dark brown, with one row of interfrontal setulae along fronto-orbital plate. Pedicel brown. First flagellomere grey yellow. Face grey yellow, facial carina not prominent. Clypeus grey yellow. Gena: narrow and linear, yellowish. Postgena yellow. Vibrissa prominent; other orals small. Palpus slender, basally grey yellow, distally black, with a few small setae along ventral margin. Occiput glossy, brownish black.

Thorax: yellow, with brown patches and silvery pollinosity. Postpronotal lobe pale yellow, with one long and a few short setae. Acrostichal setulae in approximately ten irregular rows. Prescutellar setae one pair. Anepisternum glabrous. Scutellum usually concolorous with thorax, with one obscure patch. Basal scutellar setae divergent; apicals cruciate.

Wing: brownish. Basal medial-cubital crossvein absent; C₁ setae 2, indistinctly differentiated. R₂₊₃:

slightly curved to costa at tip. R_{4+5} and M_1 nearly parallel. Halteres grey white.

Legs: yellow. Fore femur with two or three irregular rows of long setae on posterior surface. Preapical and apical dorsal setae present on all tibiae. Fore- and mid first tarsomeres each as long as other tarsomeres combined; hind first tarsomere as long as the three succeeding tarsomeres together.

Abdomen: Males tergites 1–5 with dense silvery white pollinosity, and with brown to dark brown coloration. Females yellow, with distinct pattern (Lin & Wheeler, 1972; Fig. 5B). Sternites usually grey yellow.

Male terminalia: Epandrium slightly constricted mid-dorsally, broadened below, nearly entirely pubescent, except for ventral margin, with dense setae (Figs 16, 18, 20, 22). Cercus separated from epandrium, entirely pubescent, and setigerous (Figs 16, 18, 20, 22). Surstylus quadrate, with a few setae on outer surface, and small prensisetae on apical

margin (Figs 16, 18, 20, 22). Membrane between epandrium and cercus pubescent (Figs 16, 18, 20, 22). Hypandrium narrow, arched, sub-basally with one pair of somewhat triangular plates (Figs 17, 19, 21, 23). Gonopods fused to each other, forming posteromedian plate, anteriorly forming contiguous to aedeagus (basiphallus) (Figs 17, 19, 21, 23). Parameres sub-basally and apically with a few sensilla (Figs 17, 19, 21, 23). Aedeagus: short and thick, membranous tube with pubescence distally (Figs 17, 19, 21, 23).

Included species: *Paraleucophenga semiplumata* (Duda, 1939); *Paraleucophenga argentosa* (Okada, 1956); *Paraleucophenga emeiensis* Sidorenko, 1998; *Paraleucophenga invicta* (Walker, 1857); *Paraleucophenga javana* Okada, 1988; *Paraleucophenga neojavanaii* Singh & Negi, 1992; *Paraleucophenga shimai* Okada, 1988; *Paraleucophenga brevipenis* sp. nov.; *Paraleucophenga hirtipenis* sp. nov.; *Paraleucophenga longiseta* sp. nov., and *Paraleucophenga tanydactylia* sp. nov.

KEY TO ALL *PARALEUCOPHENG*A SPECIES EXAMINED, BASED ON MALE MORPHOLOGICAL DATA

1. Frons narrow, less than one third of head width; arista with long dorsal and fine ventral branches; scutellum usually with small setae along margin; katapisternal setae 3; abdominal tergites with silvery white pollinosity; cercus elongated below and slightly constricted submedially; paramere with tooth comb; aedeagal apodeme sub-basally narrow, arched, distally broadened; (genus *Paraleucophenga*).....2
2. Scutellum without setae along margin; midleg femur medially with one row of about five strong setae on anterior surface; thorax and abdominal tergites with silvery white pollinosity.....*P. shimai*
Scutellum with small setae along margin; midleg femur lacking strong setae on anterior surface; thorax and abdominal tergites with silvery pollinosity.....3
3. Fifth sternite without long setae.....4
Fifth sternite with long setae.....6
4. Paramere submedially with longitudinal teeth row.....*P. argentosa*
Paramere distally with transverse teeth row.....5
5. Brown posterior band of third tergite as broad as half of the tergite width; fifth tergite nearly entirely dark brown, with one yellow patch laterally.....*P. emeiensis*
Brown posterior band of third tergite as broad as a fifth of the tergite width; posterior band of fifth tergite as broad as two thirds of the tergite width, laterally dark.....*P. invicta*
6. First tergite silvery white; fifth sternite with long finger-like processes, lacking spinules along posterior margin.....7
First tergite dark brown; fifth sternite with a few spinules along posterior margin, lacking long finger-like processes.....8
7. Brown posterior band of second tergite submedially as broad as half of the tergite width; fifth sternite with six long, finger-like processes.....*P. javana*
Brown posterior band of second tergite as broad as a third of the tergite width; fifth sternite with 12 long, finger-like processes.....*P. tanydactylia* sp. nov.
8. Fifth sternite with 22–23 long and strong setae; gonopods subapically with one sclerotized right process; aedeagal apodeme distally with one sclerotized right process.....*P. brevipenis* sp. nov.
Fifth sternite with 16–18 long and strong setae; gonopods subapically with one sclerotized left process.....9
9. Aedeagal apodeme distally with one sclerotized left process.....*P. hirtipenis* sp. nov.
Aedeagal apodeme distally with one sclerotized right process.....*P. longiseta* sp. nov.

KEY TO SEVEN *PARALEUCOPHENGHA* SPECIES BASED ON THE *ND2* GENE SEQUENCES

In the following key to seven *Paraleucophenga* species, each of the numbers indicates the position of a diagnostic character in the affiliated sequences, followed by its status (i.e. nucleotide type, given after a colon) and (after a slash) corresponding amino acid type (all shown in standard three-letter abbreviations; in the case of more than two types of amino acid being present, they are bracketed together, and are separated from each other using an additional slash).

1. 251: T/Met; 596: T/Ile; 653: A/Tyr; 671: G/Ser; 689: T/Phe; 827: C/Thr; 839: T/Val.....*P. emeiensis*
251: C/(Ser/Thr); 596: C/Thr; 653: T/Phe; 671: A/Asn; 689: A/His; 827: T/Met; 839: G/Ser.....2
2. 266: T/Leu; 278: C/Thr; 284: G/Ser; 440: A/Asn; 608: A/Tyr; 995: T/(Met/Ile).....3
266: C/Ser; 278: T/Ile; 284: T/Met; 440: C/Thr; 608: T/Phe; 995: G/Ser.....4
3. 46: A/Thr; 223: G/Val; 452: T/Ile; 637: T/Leu; 955: A/Met; 1019: T/Met.....*P. javana*
46: G/Ala; 223: A/Ile; 452: C/Thr; 637: A/Met; 955: G/Val; 1019: C/Thr.....*P. tanydactylia* sp. nov.
4. 106: G/Val; 275: G/Ser; 682: A/Thr; 926: A/Asn; 967: T/Phe; 1015: A/Ile.....5
106: A/Ile; 275: A/Asn; 682: T/Ser; 926: T/Phe; 967: A/Met; 1015: T/Phe.....6
5. 452: C/Thr; 799: T/Ser; 809: A/Asn; 883: G/Ala; 905: T/Phe.....*P. brevipennis* sp. nov.
452: T/Ile; 799: A/Thr; 809: G/Ser; 883: T/Ser; 905: A/Tyr.....*P. hirtipennis* sp. nov.
6. 70: G/Ala; 205: A/Thr; 314: G/Ser; 604: G/Val; 932: C/Ser; 935: A/Lys; 941: C/Thr.....*P. longiseta* sp. nov.
70: T/Ser; 205: T/Ser; 314: C/Thr; 604: A/Met; 932: T/Leu; 935: G/Ser; 941: A/Asn.....*P. argentosa*

PARALEUCOPHENGHA ARGENTOSA (OKADA, 1956)

(FIGS 1, 9)

Leucophenga (*Trichiaspiphenga*) *argentosa* Okada, 1956: 24 (synonymized as *P. invicta* by Okada, 1988: 620).

Paraleucophenga shanyinensis Chen & Toda, 1994: 71 syn. nov.

Specimens examined: Japan: paratypes, two males, one female, Setagawa, Tokyo, 2.iv.1954, T. Okada (NSMT); two males, Ueinohara, Yamanashi, 12.viii.2002, ex. around human eyes, H.W. Chen; two males, one female, Tomochi, Kumamoto, 17.viii.2002, ex. around human eyes, H.W. Chen. China: one male, Huajiang, Guilin, Guangxi, 9.ix.2001, H. Watabe.

Distribution: Japan (Honshu and Kyushu) and China (Anhui, Zhejiang, and Guangxi).

Remarks: Okada (1988) checked a specimen of *P. invicta* (Walker, 1857) from the type locality (Taiwan), and thought that *P. argentosa* was a synonym of *P. invicta*. After examining these specimens, we confirmed that the two forms are actually different species. In fact, Okada (1988) mistook the anteroventral processes of the gonopod for the paramere, and thus concluded that *P. argentosa* was a synonym of *P. invicta*. On the other hand, no morphological diagnostic character has been found to discern between *P. argentosa* and *P. shanyinensis*, thereby supporting *P. shanyinensis* as a new synonym.

PARALEUCOPHENGHA EMEIENSIS SIDORENKO, 1998

(FIGS 2, 10)

Specimens examined: China: holotype male, Emeishan, Sichuan, 550 m a.s.l., M.J. Toda (SEHU). China: three males, two females, Wuyishan, Fujian, 700 m a.s.l., 17.viii.2001, on tree trunks, H.W. Chen; two males, Guanshan, Yifeng, Jiangxi, 500 m a.s.l., 8.vi.2004, ex. around human eyes, J.J. Gao; one male, Nonggang, Longzhou, Guangxi, 550 m a.s.l., 5.vi.1993, ex. around human eyes, Y.S. Cui; two males, Emeishan, Leshan, Sichuan, 1700 m a.s.l., 4.vii.2007, ex. around human eyes, H.Z. Cao and J.J. Jiang; one male, Fengtongzhai, Baoxing, Sichuan, 1600 m a.s.l., 18.ix.2005, H.W. Chen; one male, Leigongshan, Kaili, Guizhou (~26°24'N, 108°19'E), 800 m a.s.l., 16.vii.2000, ex. around human eyes, J.J. Gao; five males, Menglun, Xishuangbanna, Yunnan, 14.ix.2002, 24–26.xii.2004, 17–20.iv.2007, 600–900 m a.s.l., ex. around human eyes, H.W. Chen and J.J. Gao.

Distribution: China (Fujian, Jiangxi, Guangxi, Sichuan, Guizhou, and Yunnan).

PARALEUCOPHENGHA INVICTA (WALKER, 1857)

(FIG. 3)

Helomyza invicta Walker, 1857: 130 (Borneo?).

Leucophenga (*Trichiaspiphenga*) *invicta*: Duda, 1924: 185 (Taiwan).

Paraleucophenga invicta: Lin & Wheeler, 1972: 248 (Taiwan; Nepal); Okada, 1988: 620 (Taiwan; Malaysia, Sarawak).

Paraleucophenga trisetata Hendel, 1914: 114 (Taiwan).

Specimens examined: China: one male, one female, Kuantsuling, Tainan, Taiwan, 6.vi.1965, T. Saigusa

Figures 1–8. Abdominal tergite patterns in males. 1, *Paraleucophenga argentosa* (Okada, 1956); 2, *Paraleucophenga emeiensis* Sidorenko, 1998; 3, *Paraleucophenga invicta* (Walker, 1857); 4, *Paraleucophenga javana* Okada, 1988; 5, *Paraleucophenga shimai* Okada, 1988; 6, *Paraleucophenga brevipennis* sp. nov.; 7, *Paraleucophenga longiseta* sp. nov.; 8, *Paraleucophenga anydactylia* sp. nov.

(NSMT). Malaysia: one male, Sarawak, 25–27. vi.1975, H. Shima (NSMT).

Distribution: Taiwan; Nepal (Kathmandu); Malaysia (Sarawak); Indonesia (Java and Sumatra).

PARALEUCOPHENGHA JAVANA OKADA, 1988
(FIGS 4, 11)

Paraleucophenga javana Okada, 1988: 621.

Specimens examined: Holotype male and one male paratype: Indonesia, Tugu, Java, 17–30.vii.1971, T. Okada (NSMT). China: one male, Dinghushan, Zhaoqing, Guangdong, 6.v.2005, M.F. Xu; three males, Nonggang, Longzhou, Guangxi, 4.vi.1993, 23.viii.2004, ex. around human eyes, Y.S. Cui and

H.W. Chen; two males, Menglun, Xishuangbanna, Yunnan, 12.viii.2002, M.J. Toda.

Distribution: China (Guangdong, Guangxi, and Yunnan), Indonesia (Java).

PARALEUCOPHENGHA SHIMAI OKADA, 1988 (FIG. 5)
Paraleucophenga shimai Okada, 1988: 621.

Specimens examined: Holotype male and three male paratypes: Thailand, Erawin Waterfall, 10.x.1975, H. Shima (NSMT). China: two males, Nonggang, Longsheng, Guangxi, 7.vii.1993, around human eyes, Y.S. Cui.

Distribution: China (Guangxi), Thailand.

Figures 9–15. Abdominal fifth sternite in males. 9, *Paraleucophenga argentosa* (Okada, 1956); 10, *Paraleucophenga emeiensis* Sidorenko, 1998; 11, *Paraleucophenga javana* Okada, 1988; 12, *Paraleucophenga brevipenis* sp. nov.; 13, *Paraleucophenga hirtipenis* sp. nov.; 14, *Paraleucophenga longiseta* sp. nov.; 15, *Paraleucophenga tanydactylia* sp. nov. Scale bars: 0.1 mm.

***PARALEUCOPHENG A BREVIPENIS* SP. NOV.**

(FIGS 6, 12, 16, 17)

Specimens examined: Holotype male, China: Nanling, Ruyuan, Guangdong, 2.v.2004, around human eyes, H.W. Chen (SCAU). Paratype: two males, same data as the holotype except for 1.x.2004, M.F. Xu (SCAU) and 2.vii.2007, H.W. Chen (SCAU).

Etymology: A combination of the Latin words breviculus and penis, referring to the shorter aedeagus.

Description: Male. Abdominal first tergites brown; second to sixth tergites with brown bands. Fifth sternite with c. 22–24 long setae and a few spinules along posterior margin (Fig. 12).

Male terminalia. Surstylus with eight prenisetae near inner surface (Fig. 16). Paramere basally and

apically each with four or five sensilla, submedially with 16–18 teeth (Fig. 17). Gonopods subapically with one sclerotized right process (Fig. 17). Aedeagal apodeme distally with one sclerotized right process (Fig. 17).

Measurements: BL, 4.56 mm in the holotype (4.55 mm in 1 male paratype); ThL, 2.30 mm (2.15 mm); WL, 4.60 mm (4.15 mm); WW, 2.00 mm (1.90 mm).

Indices: arb, 10/9–10; avd, 0.20 (0.20); adf, 1.25 (1.83); flw, 2.63 (3.17); FW/HW, 0.27 (0.27); ch/o, 0.05 (0.04); prorb, 0.42 (0.41); rcorb, 0.54 (0.74); vb, 0.24 (0.21); dcl, 0.49 (0.52); presctl, 0.39 (0.43); sctl, 1.22 (1.36); sterno, 0.38 (0.43); orbito, 1.20 (1.09); dcp, 0.29 (0.28); sctlp, 1.33 (1.25); C, 5.00 (5.45); 4c, 0.57 (0.50); 4v, 1.52 (1.45); 5x, 0.80 (0.93); ac, 1.20 (1.13); M, 0.38 (0.41); C3F, 0.53 (0.55).

Figures 16–19. 16, 17, *Paraleucophenga brevipennis* sp. nov. ♂; 18, 19, *Paraleucophenga hirtipenis* sp. nov. ♂. 16, 18, epandrium (epand), cercus (cerc) and surstylus (sur); 17, 19, hypandrium (hypd), paramere (pm), gonopod (gon), aedeagus (aed), and aedeagal apodeme (aed a). Scale bars: 0.1 mm.

***PARALEUCOPHENG*A *HIRTIPENIS* SP. NOV.**

(FIGS 13, 18, 19)

Specimens examined: Holotype male: China: Shibilianshan, Fuyuan, Qujing, Yunnan, 1.v.2006, ex. around human eyes, M.F. Xu (SCAU). Paratype: one male, same data as holotype (SCAU).

Etymology: A combination of the Latin words *hirtus* and *penis*, referring to the aedeagus with dense pubescence.

Description: Male. Abdominal first tergites brown; second to sixth tergites with brown bands (Fig. 6). Fifth sternite with 16–18 long setae and a few spinules along posterior margin (Fig. 13).

Male terminalia. Surstylus with seven prenisetae near inner surface (Fig. 18). Paramere basally and

apically each with four or five sensilla, and submedially with 17–18 teeth (Fig. 19). Gonopods subapically with one sclerotized left process (Fig. 19). Aedeagal apodeme distally with one sclerotized left process (Fig. 19).

Measurements: BL, 4.60 mm in the holotype (4.55 mm in one male paratype); ThL, 2.30 mm (2.25 mm); WL, 4.45 mm (4.65 mm); WW, 2.00 mm (2.00 mm).

Indices: arb, 10/9–10; avd, 0.20 (0.20); adf, 1.88 (1.63); flw, 2.50 (2.63); FW/HW, 0.24 (0.29); ch/o, 0.06 (0.04); prorb, 0.50 (0.44); rcorb, 0.69 (0.70); vb, 0.32 (0.29); dcl, 0.51 (0.53); presctl, 0.38 (0.47); sctl, 1.13 (1.18); sterno, 0.76 (0.54); orbito, 1.04 (1.10); dcp, 0.36 (0.36); sctlp, 1.36 (1.36); C, 4.83 (4.69); 4c, 0.55 (0.54); 4v, 1.41 (1.50); 5x, 1.00 (0.90); ac, 1.20 (1.30); M, 0.41 (0.38); C3F, 0.52 (0.47).

Figures 20–23. 20, 21, *Paraleucophenga longiseta* sp. nov. ♂; 22, 23, *Paraleucophenga tanydactylia* sp. nov. ♂. 20, 22, eandrium, cercus, and surstylus; 21, 23, hypandrium, paramere, gonopod, aedeagus, and aedeagal apodeme. Scale bars: 0.1 mm.

***PARALEUCOPHENGIA LONGISETA* SP. NOV.**

(FIGS 7, 14, 20, 21)

Specimens examined: Holotype male: China, Leigongshan, Kaili, Guizhou, 800 m, 16.vii.2000, ex. around human eyes, H.W. Chen (SCAU). Paratypes: one male, same data as holotype (SCAU); one male, Guanshan, Yifeng, Jiangxi, ex. around human eyes, H.W. Chen (SCAU); two males, two females, Maoershan, Guangxi, 19.x.2004, on tree trunks, H.W. Chen (one male and one female in SEHU, one male and one female in SCAU); four males, Emeishan, Leshan, Sichuan (~29°44'N, 103°30'E), 1.vii.2007, ex. around human eyes, H.W. Chen (two males in KIZ, two males in SCAU).

Etymology: A combination of the Latin words *longus* and *seta*, referring to the fifth sternite with long setae.

Description: Male. Thorax, pleura, and scutellum brown. Scutellum with three small setae: one of them before the basal setae, and the others between the basal and the apical setae. Abdominal first tergites brown; second to sixth tergites with brown bands (Fig. 7). Fifth sternite with c. 18–20 long setae and a few spinules along posterior margin (Fig. 14).

Male terminalia. Surstylus with six prenisetae near inner surface (Fig. 20). Paramere each with five or six sensilla basally and apically (Fig. 21). Gonopod subapically with one sclerotized left process (Fig. 21). Aedeagal apodeme distally with one sclerotized right process (Fig. 21).

Measurements: BL, 4.96 mm in the holotype (4.30 mm in one male paratype); ThL, 2.14 mm (2.20 mm); WL, 4.30 mm (4.00 mm); WW, 1.58 mm (1.90 mm).

Indices: arb, 10/10–11; avd, 0.20 (0.19); adf, 2.20 (1.83); flw, 2.30 (3.50); FW/HW, 0.30 (0.28); ch/o, 0.05 (0.06); prorb, 0.60 (0.58); rcorb, 0.70 (0.62); vb, 0.20 (0.24); dcl, 0.60 (0.57); presctl, 0.65 (0.34); sctl, 1.20 (1.18); sterno, 0.75 (0.72); orbito, 1.00 (1.10); dcp, 0.25 (0.37); sctlp, 1.50 (1.14); C, 5.50 (5.36); 4c, 0.44 (0.50); 4v, 1.31 (1.36); 5x, 0.87 (1.13); ac, 1.23 (1.38); M, 0.81 (0.41); C3F, 0.38 (0.52).

***PARALEUCOPHENG* *TANYDACTYLIA* SP. NOV.**

(FIGS 8, 15, 22, 23)

Specimens examined: Holotype male: China, Mengyang, Xishuangbanna, Yunnan, 13.ix.2002, on tree trunks, H.W. Chen (SCAU). Paratype: one male, same data as holotype (SCAU).

Etymology: A combination of the Greek words *tany-*stys and *dactylos*, referring to the fifth sternite with finger-like processes.

Description: Male. Thoracic dark brown; pleura pale yellow, with large brown patches; scutellum brown, pale yellow along margin, with three small setae around basal setae. Abdominal first tergites entirely silvery white; second and sixth tergites with brown bands (Fig. 8). Fifth sternite with *c.* 12 long strong setae and 12 finger-like processes (Fig. 15).

Male terminalia. Surstylus with about eight prensistae near inner surface (Fig. 22). Paramere basally strongly bulged, with dense sensilla, apically with dense sensilla and two rows of *c.* 23–25 teeth (Fig. 23). Gonopod distally broadened, forming one pair of curved, lobe-shaped, sclerotized processes (Fig. 23).

Measurements: BL, 4.80 mm in the holotype (4.82 mm in one male paratype); ThL, 1.78 mm (1.80 mm); WL, 4.20 mm (4.12 mm); WW, 1.70 mm (1.67 mm).

Indices: arb, 10/9–10; avd, 0.20 (0.20); adf, 2.50 (2.40); flw, 2.20 (2.20); FW/HW, 0.30 (0.30); ch/o, 0.04 (0.04); prorb, 0.55 (0.55); rcorb, 0.55 (0.60); vb, 0.20 (0.20); dcl, 0.55 (0.55); presctl, 0.55 (0.55); sctl, 1.20 (1.20); sterno, 0.80 (0.80); orbito, 1.10 (1.10); dcp, 0.25 (0.25); sctlp, 1.50 (1.50); C, 5.71 (5.40); 4c, 0.42 (0.44); 4v, 1.21 (1.18); 5x, 0.75 (0.81); ac, 1.08 (1.15); M, 0.39 (0.38); C3F, 0.57 (0.53).

RESULTS OF THE PHYLOGENETIC ANALYSIS

DATA ANALYSIS

The alignment of all of the *ND2* sequences is 1035 nucleotide positions in length. The average nucleotide

frequencies across species (*A* = 35.6%, *C* = 11.6%, *G* = 7.7%, and *T* = 45.2%) deviate greatly from the equilibrium of 25% for each nucleotide type, showing an *A* + *T*-rich pattern.

The among-lineage base composition homogeneity is not rejected by the χ^2 test. The nucleotide frequencies were similar among species ($\chi^2 = 13.9$, d.f. = 45, *P* = 1.00). The test of substitution saturation indicates that observed *I*_{ss} was significantly lower than *I*_{ss.c} (*I*_{ss} = 0.2223, and *I*_{ss.c} = 0.7683 for a symmetrical tree and 0.5747 for an extreme asymmetrical tree), indicating that the level of homoplasy in the *ND2* sequences data is low.

The uncorrected pairwise genetic distances for *ND2* sequences ranged from 0 to 0.194 (*P. tanydactylia* sp. nov. vs. *P. emeiensis*-SC or *P. emeiensis*-YN). The two sequences of *P. longiseta* sp. nov. are identical, and so are those of *P. argentosa*. More or less intraspecific sequence variations are found within each of the species *P. brevipenis* sp. nov., *P. javana*, and *P. emeiensis*, with the uncorrected genetic distance (*p*-distance, i.e. the number of base differences per site) among conspecific forms ranging from 0.006 to 0.018.

PHYLOGENETIC RELATIONSHIPS

Figure 24 show the trees deduced from the *ND2* sequences (1035 sites, with 322 parsimony-informative characters). The strict consensus of the two MP trees (Fig. 24A; tree length, TL = 677; consistency index, CI = 0.7415; retention index, RI = 0.8573) shows a largely similar topology to the ML tree (Fig. 24B; $-\ln L = 4410.3499$). The confidence (posterior probability, PP) of each node in the Bayesian tree (not shown), which has identical to the ML tree, is given on the corresponding node in the ML tree. In all of these trees, the monophyly of the genus *Paraleucophenga* is well supported with respect to the out-group species (MP and ML BPs = 100; Bayesian PP = 1.00), and each of the species represented by multiple populations proved to be monophyletic with strong confidence (MP BPs = 100; ML BPs $\geq$ 88; PPs = 1.00). All of the trees indicate that *P. emeiensis* diverged first among the species of the genus *Paraleucophenga*, with the rest forming a monophyletic clade (MP and ML BPs = 100; Bayesian PP = 1.00) that subsequently diverged into two subclades, one consisting of *P. javana* and *P. tanydactylia* sp. nov. (MP and ML BPs = 100; PP = 1.00), and the other consisting of *P. argentosa*, *P. hirtipenis* sp. nov., *P. brevipenis* sp. nov., and *P. longiseta* sp. nov. (MP and ML BPs = 96 and 100, respectively; PP = 1.00). Two species pairs are strongly supported in the latter subclade: the *P. hirtipenis* sp. nov.–*P. brevipenis* sp. nov. pair (MP and ML BPs = 100 and 96, respec-

Figure 24. Phylogenetic trees deduced from the ND2 sequences. A, strict consensus tree of two equally parsimonious trees (677 steps). B, maximum likelihood (ML) tree. Numbers to the left of nodes in (A) and (B) indicate bootstrap proportions (%) of maximum parsimony (MP) and ML methods, respectively; those to the right of nodes in (B) indicate the Bayesian posterior probabilities (PP).

tively; PP = 1.00) and the *P. shanyinensis*–*P. argentosa* pair (MP and ML BPs = 100; PP = 1.00). However, the MP tree differs from the ML and the Bayesian trees, in that it clusters the above two species pairs with low confidence (BP = 54). In the latter two trees, the *P. shanyinensis*–*P. argentosa* pair is clustered with *P. longiseta* sp. nov. (BP = 66; PP = 0.93). Moreover, the MP tree yielded an intraspecific relationship for *P. emeiensis* as (FJ (JX (YN, SC))), which differs from those found in the ML and Bayesian trees, i.e. (YN (SC (JX, FJ))).

ABBREVIATIONS

BL, body length; ThL, thorax length; WL, wing length; WW, wing width; arb, dorsal branches/ventral branches of arista; avd, longest ventral branch/longest dorsal branch of arista in length; adf, longest dorsal branch of arista/width of first flagellomere; flw, length/width of first flagellomere; FW/HW, frontal width/head width; ch/o, maximum width of gena/maximum diameter of eye; prorb, proclinate orbital/posterior reclinate orbital in length; rorb, anterior

reclinate orbital/posterior reclinate orbital in length; vb, subvibrissal/vibrissa in length; dcl, anterior dorsocentral/posterior dorsocentral in length; presctl, prescutellar/posterior dorsocentral in length; sctl, basal scutellar/apical scutellar in length; sterno, anterior katapisternal/posterior katapisternal in length; orbito, distance between proclinate and posterior reclinate orbitals/distance between inner vertical and posterior reclinate orbital; dcp, length distance between ipsilateral dorsocentrals/cross distance between anterior dorsocentrals; sctlp, distance between ipsilateral scutellars/cross distance between apical scutellars; C, second costal section between subcostal break and R_{2+3} /third costal section between R_{2+3} and R_{4+5} ; 4c, third costal section between R_{2+3} and R_{4+5}/M_1 between r-m and dm-cu; 4v, M_1 between dm-cu and wing margin/ M_1 between r-m and dm-cu; 5x, CuA_1 between dm-cu and wing margin/dm-cu between M_1 and CuA_1 ; ac, third costal section between R_{2+3} and R_{4+5} /distance between distal ends of R_{4+5} and M_1 ; M, CuA_1 between dm-cu and wing margin/ M_1 between r-m and dm-cu; C3F, length of heavy setation in third costal section/(length of heavy setation in third costal

section + length of light setation in third costal section).

DISCUSSION

SYNONYM

All of the phylogenetic trees (Fig. 24) suggest a sister relationship between the two previously recognized species *P. shanyinensis* and *P. argentosa*, with high confidence. The divergence in *ND2* sequences between *P. shanyinensis* and *P. argentosa* (P distance = 0.019) is comparable with those among the three populations of *P. emeiensis* (average p-distance = 0.015), whereas this divergence is relatively smaller than the evaluated *ND2* sequence divergence between populations of *Drosophila* (*Drosophila*) *tsigana* Burla and Gloor, 1952 from China and Japan (Mean p-distance = 0.036; sequences from Wang *et al.*, 2006). Some larger divergences in *ND2* sequences can be evaluated among the closely related species *Drosophila* (*Sophophora*) *subobscura* Collin, 1936, *Drosophila* (*Sophophora*) *ambigua* Pomini, 1940, and *Drosophila* (*Sophophora*) *guanche* Monclús, 1976 (mean p-distance = 0.045; sequences from Gao *et al.*, 2007). As no morphological diagnostic character has been found to distinguish *P. shanyinensis* from *P. argentosa*, we tentatively regard *P. shanyinensis* Chen & Toda (1994) as a junior synonym of *P. argentosa* Okada (1956), and will hereafter suppress the name *shanyinensis* in this present paper.

ENANTIOMORPHISM IN GENITALIA STRUCTURE

The inner genitalia of the two species *P. brevipennis* sp. nov. and *P. hirtipennis* sp. nov. seem to be enantiomorphous. Such a phenomenon was previously reported in other drosophilid species: for example, in *Phortica speculum* (Máca & Lin, 1993) the phallic organs have a reversed structural arrangement in both the holotype and the paratype (Máca & Lin, 1993). Moreover, mirror-image variation was found in the asymmetric structure of the vertical process of gonopods for *Phortica brachychaeta* Chen & Toda, 2005 (Chen, Toda & Gao, 2005). Our studies elsewhere have revealed that the *ND2* nucleotide sequences are identical between the different enantiomorphism types in either *Phortica speculum* or *Phortica brachychaeta* (X.L. Wang, H.L. Cao, S. Prigent, J.J. Gao, H. Watabe, Y.P. Zhang, & H.W. Chen, unpubl. data).

PHYLOGENETIC RELATIONSHIPS AMONG SPECIES OR SPECIES CLUSTERS

The MP tree yielded a branching order among *P. longiseta* sp. nov., the *P. shanyinensis*–*P. argentosa*

pair, and the *P. hirtipennis* sp. nov.–*P. brevipennis* sp. nov. pair that differs from those in the ML (and in the Bayesian) tree(s). However, neither of the branching orders seems to be reliable, as the corresponding node confidences are relatively low. It could be noted in the two MP trees (not shown) that the branch leading to the *P. shanyinensis*–*P. argentosa* and *P. hirtipennis* sp. nov.–*P. brevipennis* sp. nov. pairs is quite a bit shorter than those leading to the other species (or species clusters), and so is the branch leading to *P. shanyinensis*–*P. argentosa* and *P. longiseta* in the ML tree (Fig. 24B). This means that these lineages diverged from one another in very short succession. No essential homoplasy was found in our sequence data, so it is likely that the phylogenetic difficulty associated with the above lineages is the result of a different assortment of the ancestral polymorphism (O’Higin *et al.*, 2002).

We used the program Spectrum 2.3 (Charleston, 1998), which is capable of showing both the support and the conflict for different groupings, to perform a spectral analysis. According to the ‘Evaluate Bipartition’ calculation, the spectral analysis lent good support to the nodes common to the MP and ML trees. The support for (*P. argentosa*, *P. hirtipennis* sp. nov., *P. brevipennis* sp. nov., and *P. longiseta* sp. nov.) as a split (0.01277) was high, with low normalized conflict (0.00374). However, different support and conflict values were found when the two splits, (*P. argentosa* and *P. longiseta* sp. nov.) in the ML tree and (*P. argentosa*, *P. hirtipennis* sp. nov., and *P. brevipennis* sp. nov.) in MP tree, were divided. The support for the former as a split (0.00196) was relatively higher than the support for the latter (0.00098); moreover, the former value of the normalized conflict (0.00170) was relatively lower than the latter (0.00442). Support for (*P. hirtipennis* sp. nov., *P. brevipennis* sp. nov., and *P. longiseta* sp. nov.) as a split (0.00098) was lower than the normalized conflict (0.00272). Based on the above results, the relationship reflected in the ML tree, i.e. (*P. argentosa* and *P. longiseta* sp. nov.) as a split, should be considered as more reliable.

MORPHOLOGICAL VS. MOLECULAR AFFINITIES

In this study, the sibling species relationship between *P. hirtipennis* sp. nov. and *P. brevipennis* sp. nov. or between *P. tanydactylia* sp. nov. and *P. javana* were strongly supported. These close relationships are congruent with the special morphological similarity between each of the species pairs: *P. tanydactylia* sp. nov. resembles *P. javana* in the fifth sternite with long, finger-like processes, and paramere basally and apically with sensilla, subapically with tooth row(s); *P. hirtipennis* sp. nov. resembles *P. brevipennis* sp.

nov. in the fifth sternite with long, strong setae and a few spinules along posterior margin, and in gonopods subapically with one sclerotized process, and aedeagal apodeme distally with one sclerotized process.

Some prominent morphological characters appear to suggest incongruent interspecific relationships compared with the molecular data: with respect to the morphological trait of the fifth sternite, *P. argentosa* appears to be closer to *P. emeiensis* than to *P. longiseta* sp. nov., and *P. longiseta* sp. nov. appears to be closer to *P. hirtipenis* sp. nov./*P. brevipenis* sp. nov. than to *P. argentosa*; whereas with respect to the colour patterns of male abdominal tergites, *P. longiseta* sp. nov. and *P. brevipenis* sp. nov. are closer to each other than either is to the other species.

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