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## Research Article

The subgenus *Ashima* (Diptera, Drosophilidae, *Phortica*) from China, with DNA barcoding and descriptions of three new speciesKEYING AN<sup>1</sup>, HUILUO CAO<sup>2</sup>, XUELIN WANG<sup>3</sup> & HONGWEI CHEN<sup>1</sup><sup>1</sup>Department of Entomology, South China Agricultural University, Tianhe, Guangzhou, 510642, China<sup>2</sup>Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China<sup>3</sup>Shenzhen Agricultural Science and Technology Promotion Center, Futian, Shenzhen, 518040, China

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In the present study, three new species found in Yunnan, Southwest China were described as members of the subgenus *Phortica* (*Ashima*): *P. (A.) haba* An & Chen, sp. nov., *P. (A.) montipagana* An & Chen, sp. nov. and *P. (A.) qingsongii* An & Chen, sp. nov. Barcode sequences (partial sequences of the mitochondrial *COI* gene) were collected from 61 specimens of 16 known and the above-mentioned three new *Ashima* species. The intra- and interspecific pairwise K-2P (Kimura two-parameter) *COI* distances were analysed and a phylogenetic tree was constructed based on the barcode sequences. Species delimitation in this subgenus was supported by integrating barcodes with morphological information, in particular for the three new species, considered to be cryptic species. In addition, the diversification of lineages in the subgenus *Ashima* was proposed to occur in southern China and adjacent areas, suggesting specific adaptation of *Ashima* species to the high plateau environments.

<http://zoobank.org/urn:lsid:zoobank.org:pub:69A81D0E-5993-426D-9B59-A2AC5E52BD84>

**Key words:** biodiversity, cryptic species, drosophilid, morphology, taxonomy

## Introduction

Up to the present, a total of 31 species of the subgenus *Ashima* (the genus *Phortica* Schiner, 1862) has been known worldwide (Chen & Máca, 2012; Máca, 2003); seven from the Afrotropical region, the rest from the Oriental region, including 16 species from southern China. Recently, based on DNA sequence data of two mitochondrial (cytochrome oxidase subunit I, *COI*, NADH dehydrogenase subunit 2, *ND2*) and one nuclear (*28S rRNA*) genes, phylogenetic relationships within the African and Asian species of the genus *Phortica* were analysed, suggesting that southern China might be an important area for the diversification of the subgenus *Ashima* (Cao *et al.*, 2011). Further field surveys revealed higher *Ashima* species diversity in southern China (Chen & Máca, 2012).

The DNA barcoding method has been recently widely used for species delimitation in varied groups of insects, including drosophilids (Gao & Chen, 2014; Li *et al.*, 2014; Padial, Miralles, De la Riva, & Vences, 2010; Rach,

DeSalle, Sarkar, Schierwater, & Hadrys, 2008; Yang *et al.*, 2012; Yassin, Cappy, Madi-Ravazzi, Ogereau, & David, 2008; Yassin, Markow, Narechania, O'Grad, & DeSalle, 2010; Zhang, Xu, & Chen, 2012; Zhang, Tsaur, & Chen, 2012, 2014; Zou, Li, Kong, Yu, & Zheng, 2011). With the aid of DNA barcoding, high species diversity in the genus *Leucophenga* (Drosophilidae, Steganinae) was successfully recognized and numerous cryptic species in *Leucophenga* were found (Huang, Li, & Chen, 2013a, 2014; Huang, Li, Gao, & Chen, 2013b; Su, Lu, & Chen, 2013). In the present study, we constructed the phylogenetic relationship in *Ashima* based on barcode (*COI*) sequences, and delimited the species using both morphological and molecular data.

## Materials and methods

## Materials and morphological terminology

Specimens were captured while hovering around people in forests or resting on tree trunks. Most of the specimens examined were males. Specimens identified as *Ashima* were preserved in 75% ethanol before the following procedures. Freeze-dried specimens were pinned after

morphological examinations and identifications were made in the laboratory. Type specimens were deposited in the Department of Entomology, South China Agricultural University (SCAU), Guangzhou, China. Definitions of measurements, indices and abbreviations are referred to by Chen and Toda (2001) and Zhang and Toda (1992).

The *COI* sequences of 61 specimens of 18 *Ashima* species from China were employed, including the sequences for 13 individuals of eight species being determined in the present study (Table 1). Three species from the genus *Apsiphortica* and one species of the subgenus *Phortica*

(*Alloparadisa*) were used as outgroup taxa, and their *COI* sequences are mostly determined here, with the remaining sequences collected elsewhere (Table 1).

## DNA extraction and gene sequencing

Total DNA was extracted from the abdominal tissue of a single individual after the dissection of the genitalia, using the TIANGEN™ DNA extraction kit (Tiangen, China). The *COI* fragments were amplified using the cycle protocol by Zhao, Gao, and Chen (2009). The primer pair for PCR/

**Table 1** Details of the samples using in the DNA and accession numbers of the *COI* sequences.

Species	Collection	BOLD process ID	GenBank accession numbers
<i>Ap. lini</i> (Okada, 1971)	Dabang, Jiayi, Taiwan	BDORJ003-13	KJ082995
<i>Ap. longiciliata</i> Cao & Chen, 2007	Menglun, Mengla, Yunnan	————	HQ011959 <sup>b</sup>
<i>Ap. longiciliata</i> Cao & Chen, 2007	Hesong, Menghai, Yunnan	BDORJ001-13	KJ082996
<i>Ap. melanogaster</i> Chen, 2007	Menglun, Mengla, Yunnan	BDORJ002-13	KJ082997
<i>P. (Al.) helva</i> Chen & Gao, 2008	Muyiji Park, Ximeng, Yunnan	BDORJ004-13	KJ083007
<i>P. (Al.) helva</i> Chen & Gao, 2008	Menglun, Mengla, Yunnan	————	EU500837 <sup>a</sup>
<i>P. (As.) afoliolata</i> Chen & Toda, 2005	Jianfengling, Ledong Hainan	————	HQ011968 <sup>b</sup>
<i>P. (As.) brachychaeta</i> Chen & Toda, 2005	Dinghushan, Zhaoqing, Guangdong	BDORJ005-13	KJ082999
<i>P. (As.) brachychaeta</i> Chen & Toda, 2005	Yixiang, Pu'er, Yunnan	BDORJ006-13	KJ082998
<i>P. (As.) foliacea</i> (Tsacas & Okada, 1983)	Wugongshan, Kaohsiung, Taiwan	BDORJ007-13	KJ083000
<i>P. (As.) foliiseta</i> Duda, 1923	Dinghushan, Zhaoqing, Guangdong	————	HQ011970 <sup>b</sup>
<i>P. (As.) foliiseta</i> Duda, 1923	Dinghushan, Zhaoqing, Guangdong	BDORJ008-13	KJ083002
<i>P. (As.) foliiseta</i> Duda, 1923	Chebaling, Xingshi, Guangdong	BDORJ009-13	KJ083001
<i>P. (As.) foliisetoides</i> Chen & Toda, 2005	Jianfengling, Ledong Hainan	————	HQ011971 <sup>b</sup>
<i>P. (As.) glabra</i> Chen & Toda, 2005	Nanling, Shaoguan, Guangdong	————	HQ011978 <sup>b</sup>
<i>P. (As.) glabra</i> Chen & Toda, 2005	Dinghushan, Guangdong	BDORJ011-13	KJ083003
<i>P. (As.) glabra</i> Chen & Toda, 2005	Sanchahe, Xishui, Guizhou	BDORJ012-13	KJ083004
<i>P. (As.) glabra</i> Chen & Toda, 2005	Maoershan, Guilin, Guangxi	BDORJ013-13	KJ083005
<i>P. (As.) huiluoi</i> Cheng & Chen, 2008	Jizushan, Binchuan, Yunnan	————	HQ011975 <sup>b</sup>
<i>P. (As.) longipenis</i> Chen & Gao, 2005	Wulingshan, Jingdong, Yunnan	————	HQ011979 <sup>b</sup>
<i>P. (As.) longipenis</i> Chen & Gao, 2005	Jizushan, Binchuan, Yunnan	BDORJ014-13	KJ083009
<i>P. (As.) longipenis</i> Chen & Gao, 2005	Bailongshan, Xingyi, Guizhou	BDORJ015-13	KJ083010
<i>P. (As.) longipenis</i> Chen & Gao, 2005	Sanchage, Xishui, Guizhou	BDORJ016-13	KJ083011
<i>P. (As.) longipenis</i> Chen & Gao, 2005	Hesong, Menghai, Yunnan	BDORJ017-13	KJ083008
<i>P. (As.) longipenis</i> Chen & Gao, 2005	Jizushan, Binchuan, Yunnan	BDORJ018-13	KJ083012
<i>P. (As.) nudiarista</i> Cheng & Chen, 2008	Menglun, Mengla, Yunnan	————	HQ011972 <sup>b</sup>
<i>P. (As.) nudiarista</i> Cheng & Chen, 2008	Beibeng, Motuo, Xizang	BDORJ019-13	KJ083019
<i>P. (As.) nudiarista</i> Cheng & Chen, 2008	Zhengxing, Jinggu, Yunnan	BDORJ020-13	KJ083020
<i>P. (As.) nudiarista</i> Cheng & Chen, 2008	Zhengxing, Jinggu, Yunnan	BDORJ021-13	KJ083018
<i>P. (As.) nudiarista</i> Cheng & Chen, 2008	Muyiji Park, Ximen, Yunnan	BDORJ022-13	KJ083021
<i>P. (As.) pavriarista</i> Cheng & Chen, 2008	Yixiang, Pu'er, Yunnan	BDORJ023-13	KJ083023
<i>P. (As.) pavriarista</i> Cheng & Chen, 2008	Bailongshan, Xingyi, Guizhou	BDORJ024-13	KJ083022
<i>P. (As.) pavriarista</i> Cheng & Chen, 2008	Bapen, Fusui, Guangxi	————	HQ011973 <sup>b</sup>
<i>P. (As.) sagittaristula</i> Cheng & Chen, 2005	Menglun, Mengla, Yunnan	BDORJ025-13	KJ083025
<i>P. (As.) sagittaristula</i> Cheng & Chen, 2005	Menglun, Mengla, Yunnan	————	HQ011974 <sup>b</sup>
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Guanshan, Yifeng, Jiangxi	————	EU500838 <sup>a</sup>
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Maoershan, Guilin, Guangxi	BDORJ026-13	KJ083026
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Dinghushan, Zhaoqing, Guangdong	BDORJ027-13	KJ083027
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Sanchahe, Xishui, Guizhou	BDORJ028-13	KJ083028
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Tianmushan, Linan, Zhejiang	BDORJ029-13	KJ083029

(continued)

**Table 1** (Continued)

Species	Collection	BOLD process ID	GenBank accession numbers
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Emeishan, Leshan, Sichuan	BDORJ030-13	KJ083030
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Emeishan, Leshan, Sichuan	BDORJ031-13	KJ083031
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Chebaling, Xingshi, Guangdong	BDORJ032-13	KJ083032
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Chebaling, Xingshi, Guangdong	BDORJ033-13	KJ083033
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Qinling, Foping, Shaanxi	BDORJ034-13	KJ083034
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Nanling, Shaoguan, Guangdong	BDORJ035-13	KJ083035
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Wushan, Guangzhou, Guangdong	BDORJ036-13	KJ083036
<i>P. (As.) speculum</i> (Máca & Lin, 1993)	Wuyishan, Fujian and Jiangxi	BDORJ037-13	KJ083037
<i>P. (As.) spinosa</i> Chen & Gao, 2005	Yixiang, Pu'er, Yunnan	————	HQ011977 <sup>b</sup>
<i>P. (As.) spinosa</i> Chen & Gao, 2005	Jiangfengling, Ledong, Hainan	BDORJ038-13	KJ083039
<i>P. (As.) spinosa</i> Chen & Gao, 2005	Hesong, Menghai, Yunnan	BDORJ010-13	KJ083038
<i>P. (As.) spinosa</i> Chen & Gao, 2005	Muyiji Park, Ximeng, Yunnan	BDORJ039-13	KJ083040
<i>P. (As.) spinosa</i> Chen & Gao, 2005	Niuluohe, Jiangcheng, Yunnan	BDORJ040-13	KJ083041
<i>P. (As.) spinosa</i> Chen & Gao, 2005	Hesong, Menghai, Yunnan	BDORJ041-13	KJ083042
<i>P. (As.) spinosa</i> Chen & Gao, 2005	Wangtianshu, Mengla, Yunnan	BDORJ042-13	KJ083043
<i>P. (As.) spinosa</i> Chen & Gao, 2005	Jianfengling, Ledong, Hainan	BDORJ043-13	KJ083044
<i>P. (As.) tanabei</i> Chen & Toda, 2005	Shimentai, Yingde, Guangdong	————	HQ011976 <sup>b</sup>
<i>P. (As.) tanabei</i> Chen & Toda, 2005	Menglun, Mengla, Yunnan	BDORJ044-13	KJ083045
<i>P. (As.) tanabei</i> Chen & Toda, 2005	Menglun, Mengla, Yunnan	BDORJ045-13	KJ083046
<i>P. (As.) xishuangbanna</i> Cheng & Chen, 2008	Menglun, Mengla, Yunnan	————	HQ011969 <sup>b</sup>
<i>P. (As.) haba</i> An & Chen, sp. nov.	Longtan Park, Ximeng, Yunnan	BDORJ047-13	KJ083006
<i>P. (As.) montipagana</i> An & Chen, sp. nov.	Muyiji Park, Ximeng, Yunnan	BDORJ048-13	KJ083015
<i>P. (As.) montipagana</i> An & Chen, sp. nov.	Caiyanghe, Pu'er, Yunnan	BDORJ049-13	KJ083016
<i>P. (As.) montipagana</i> An & Chen, sp. nov.	Hesong, Menghai, Yunnan	BDORJ050-13	KJ083017
<i>P. (As.) montipagana</i> An & Chen, sp. nov.	Yixiang, Pu'er, Yunnan	BDORJ051-13	KJ083013
<i>P. (As.) montipagana</i> An & Chen, sp. nov.	Baihualing, Baoshan, Yunnan	BDORJ052-13	KJ083014
<i>P. (As.) qingsongi</i> An & Chen, sp. nov.	Baihuling, Baoshan, Yunnan	BDORJ046-13	KJ083024

<sup>a</sup>*Ap.* = genus *Apsiphortica*, *P. (Al.)* = subgenus *Phortica* (*Alloparadisa*), *P. (As.)* = subgenus *Phortica* (*Ashima*); <sup>a</sup> Determined in He, X.F., Gao, J.J., Cao, H.Z., Zhang, X.L., Chen, H.W. 2009; <sup>b</sup> Determined in Cao *et al.* (2011).

sequencing was: 5'-CGCCTAAACTTCAGCCACTT-3' (Wang *et al.*, 2006) and 5'-TAAACTTCAGGGTGACAAAAATCA-3' (Folmer, Black, Hoh, Lutz, & Vrijenhoek, 1994).

### Sequence alignment and phylogenetic analysis

Obtained sequences were aligned with the ClustalW module as implemented in MEGA 5.05 (Tamura *et al.*, 2011). Aligned sequences were edited manually and a common frame length of 625 nucleotides was shared by all of the samples.

The intra- and interspecific K-2P (Kimura's two-parameter; Kimura, 1980) genetic distances were calculated in MEGA. Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian methods. The most appropriate substitution model of DNA sequences was determined according to the Akaike information criterion (AIC) (Akaike, 1974) using jModelTest v.0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). The general

time reversible (GTR) model (Lanave, Preparata, Saccone, & Serio, 1984; Rodriguez, Oliver, Marin, & Medina, 1990) with the proportion of invariable sites (0.632) and gamma distribution (GTR+I+G,  $\alpha = 0.145$ ) was used. The ML phylogenetic tree was constructed using PhyML v.3.0 (Guindon & Gascuel, 2003). The approximate likelihood-ratio test (aLRT) (Anisimova & Gascuel, 2006) and bootstrap resampling with 100 replicates were applied to estimate a statistical support for internal nodes. In addition, a Bayesian tree was deduced using the Bayesian relaxed clock as implemented in BEAST v.2.0.3 (Drummond & Rambaut, 2007). The length of the Markov chain Monte Carlo (MCMC) chain was set for 1 000 000 and sampled every 100 steps. The initial 25% of the samples were discarded as burn-in. The maximum clade credibility tree was determined using TreeAnnotator v.1.5.3 from the BEAST software package. In both ML and Bayesian trees, one *Phortica* (*Alloparadisa*) species (*P. helva*) and three species of the genus *Apsiphortica* (*A. lini*, *A. longiciliata* and *A. melanogaster*) were used as out-group taxa.

## Results

### Molecular data analyses

The length of *COI* gene sequences of all specimens ranged from 645 to 708 nucleotide sites determined in the current study. Thus, the final length of 623 nucleotides was selected for the following genetic distance and phylogenetic analyses.

The intra- and interspecific distances were calculated (Table 2). The lowest minimum interspecific divergence (0.5%) was observed between *Ashima afoliolata* and other species, as well between *P. qingsongi* and related species (0.5%), while the highest minimum interspecific divergence existed between *P. huiluoi* and the rest (13.3%) (Table 2). For the two new species in the present study, *P. haba* and *P. montipagana*, the minimum interspecific genetic distance were 2.1% and 3.0%, respectively. Regarding the maximum intraspecific divergences, the largest one was calculated within *P. tanabei* (1.5%), followed by *P. spinosa* (1.3%) and *P. glabra* (1.3%) (Table 2). As there were only two specimens collected for each in *P. brachychaeta* and *P. pevriarista*, the intraspecific divergences were not counted (Table 2).

Although three criteria were selected to construct the phylogenetic tree, topologies from these three methods were consistent with each other. Thus, only the ML tree was presented here, but the statistical supports from these three criteria were mapped on the nodes (Fig. 1). The three clades (Clades I, III, IV) within the subgenus

*Ashima*, previously recognized by Cao *et al.* (2011), were confirmed in the present phylogenetic analysis (Fig. 1). The Clade I shows robust statistical support. The Clade IV has relatively high statistical support, with exclusion of *P. tanabei*. Clade III presents remarkable support in particular deduced from Bayesian criterion shown by five species including *P. montipagana* and *P. foliacea*, not yet employed by Cao *et al.* (2011).

### Subgenus *Ashima* Chen

*Phortica* (*Ashima*) Chen in Cao *et al.*, 2011: 682; Chen and Máca, 2012: 495.

**Type species:** *Amiota* (*Phortica*) *foliiseta* Duda, 1923.

In the descriptions of the new species herein, only characters that depart from the universal description given by Chen, Gao, and Wen (2005) for the '*foliiseta* species complex' are provided for brevity.

*Phortica* (*Ashima*) *haba* An & Chen, sp. nov.  
(Figures 2–6)

HOLOTYPE: SCAU, DIP 124048, ♂ (31.3.2011).

TYPE LOCALITY: CHINA: Longtan Park, Ximeng, Yunnan, 22°37'N, 99°36'E, 1140 m a.s.l., JM Lu.

ETYMOLOGY: The name means 'the Feast of Moon' in the language of the Wa nationality in Yunnan.

**Table 2.** Divergence in *COI*-barcode sequences among and within species.

Species	Number of sequences	Intra./Min. intra./Max. intra./Mean intra. Vari.	Min. inter./Max. inter./Mean inter. Vari.
<i>P. afoliolata</i>	1	NA/NA/NA/NA	0.005/0.121/0.093±0.017
<i>P. brachychaeta</i>	2	0.016/NA/NA/NA	0.047/0.119/0.081±0.021
<i>P. foliacea</i>	1	NA/NA/NA/NA	0.035/0.133/0.093±0.028
<i>P. foliiseta</i>	3	NA/0.000/0.003/0.002±0.002	0.016/0.108/0.062±0.028
<i>P. foliisetoides</i>	1	NA/NA/NA/NA	0.032/0.123/0.085±0.021
<i>P. glabra</i>	4	NA/0.000/0.013/0.006±0.004	0.066/0.113/0.084±0.010
<i>P. huiluoi</i>	1	NA/NA/NA/NA	0.076/0.133/0.104±0.016
<i>P. longipenis</i>	6	NA/0.000/0.010/0.004±0.003	0.030/0.119/0.085±0.020
<i>P. nudiarista</i>	5	NA/0.000/0.006/0.003±0.002	0.016/0.104/0.063±0.027
<i>P. pevriarista</i>	2	0.01/NA/NA/NA	0.038/0.117/0.078±0.024
<i>P. sagittaristula</i>	2	0.005/NA/NA/NA	0.032/0.117/0.083±0.021
<i>P. saltiaristula</i>	1	NA/NA/NA/NA	0.028/0.111/0.076±0.026
<i>P. speculum</i>	14	NA/0.000/0.008/0.003±0.002	0.074/0.132/0.098±0.011
<i>P. spinosa</i>	8	NA/0.005/0.013/0.009±0.002	0.047/0.108/0.079±0.016
<i>P. tanabei</i>	4	NA/0.002/0.015/0.008±0.006	0.023/0.121/0.101±0.017
<i>P. xishuangbanna</i>	1	NA/NA/NA/NA	0.021/0.100/0.061±0.027
<i>P. haba</i>	1	NA/NA/NA/NA	0.030/0.115/0.080±0.020
<i>P. montipagana</i>	5	NA/0.000/0.000/0.000	0.050/0.119/0.094±0.017
<i>P. qingsongi</i>	1	NA/NA/NA/NA	

Intra., intraspecific distance; Min. intra., minimum intraspecific distance; Max. intra., maximum intraspecific distance; Mean intra. Vari., mean intraspecific variability; Min. inter., minimum interspecific distance; Max. inter., maximum interspecific distance; Mean inter. Vari., mean interspecific variability; NA, not applicable.

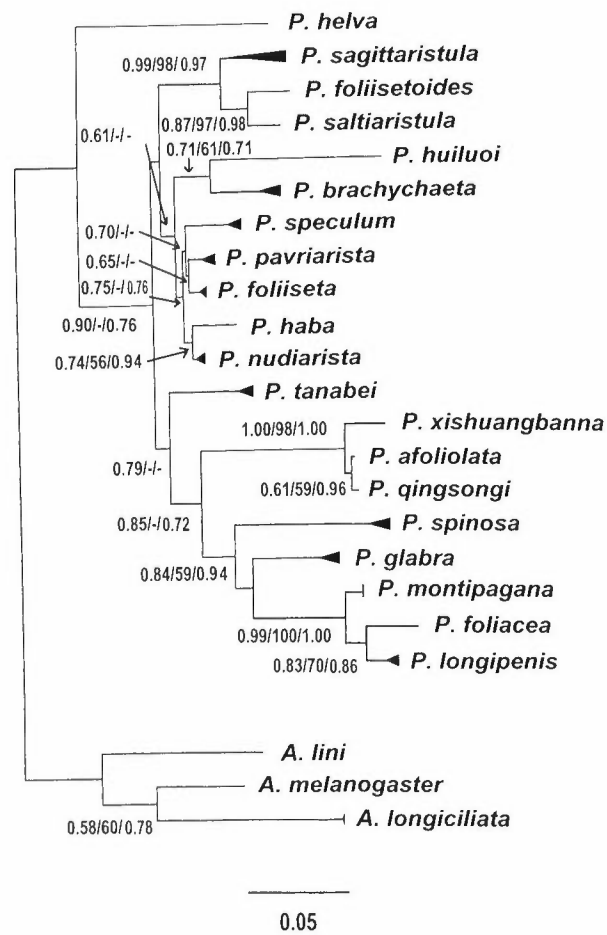


Fig. 1. Maximum-likelihood tree constructed from *COI* partial gene sequences. The aLRT values and Bayesian posteriors below 0.5 and bootstrap below 50 are not shown. Internal node labels are likelihoods, bootstraps, and posteriors, respectively. The bar below the tree indicates 0.05 substitutions per site.

**DIAGNOSIS:** This species is similar to *P. brachychaeta* and *P. nudiarista* in the arista lacking branches (Fig. 2) and the similar shape of paramere (Fig. 6), but can be distinguished from the latter two species by the gonopods (in *P. brachychaeta* and *P. nudiarista*: vertical process of gonopods nearly triangular, with 1 sclerotized projection apically; as in Chen, H. W., Gao, J. J., & Wen, S. Y. 2005, fig. 8, and in Cheng, Gao, Watabe and Chen 2008, fig. 11); in *P. haba* this process with two curved apical hooks each side.

**MEASUREMENTS:** BL = 3.33 mm in holotype, THL = 1.67 mm, WL = 3.27 mm, WW = 1.13 mm, arb = 0/0, flw = 2.67, FW/HW = 0.31, ch/o = 0.19, prorb = damaged, rcorb = 0.44, vb = 0.80, dcl = 0.56, presctl = 0.56, sctl = 1.12, sterno = 0.75, orbito = 1.83, dcp = 0.29, sctlp = 1.60, C = 2.00, 4c = 1.57, 4v = 2.64, 5x = 0.88, ac = 4.40, M = 0.50, C3F = 0.77.

## Description

Frons dark brown, with thick and dense interfrontal setae. Pedicel dark brown; first flagellomere brown. Face dark brown, with yellowish white patches on lower corners. Clypeus medially white, laterally black. Thorax black anteriorly, orange brown posteriorly and laterally. Scutellum dark brown medially, black laterally. Legs entirely yellow; fifth tarsomere of foreleg with long seta apically (as in Cao *et al.*, 2011, fig. 1D). Abdominal tergites third to fifth yellow, with broad black posterior bands uninterrupted medially; sixth tergite entirely black. All sternites more or less evenly narrow. Male terminalia: Surstylus with several setae and pubescence, distal margin of inner surface lacking prenisetae (Fig. 4). Paramere apically roundly knobbed and smooth, submedially slightly curved, basally with 2–3 sensilla (Figs 5, 6). Vertical process of gonopods asymmetrically constricted, forming 2 sclerotized, hooked projections (Fig. 5). Aedeagus lacking median rod (Fig. 5).

*Phortica* (*Ashima*) *montipagana* An & Chen, sp. nov.  
(Figs 7–11)

**HOLOTYPE:** SCAU, DIP 124049, ♂ (19.4.2010).

**TYPE LOCALITY:** CHINA: Yixiang, Pu'er, Yunnan, 22°47'N, 101°02'E, 1100–1400 m a.s.l., JJ Gao.

**PARATYPES:** SCAU, DIP 124050–51, 2♂ (19.4.2010), same data as holotype. SCAU, DIP 124052–63, 12♂ (4.4.2011), CHINA: Muyiji Park, Ximeng, Yunnan, 22°37'N, 99°37'E, 1100 m a.s.l., JM Lu, YR Su, ZF Shao, SJ Yan; SCAU, DIP 124064–75, 12♂ (12.5.2012), CHINA: Hesong, Menghai, Yunnan, 21°49'N, 100°06'E, 1700 m a.s.l., HW Chen, JJ Gao; SCAU, DIP 124123–35, 13♂ (22–24.8.2013), Baihualing, Baoshan, Yunnan, 25°27'N, 98°52'E, 1400 m a.s.l., K Lu, QS Gao.

**ETYMOLOGY:** A combination of the Latin words: mons + paganus, referring to these specimens collected from a near mountain village.

**DIAGNOSIS:** This species is similar to *P. longipennis* in the aedeagal outer membrane bearing numerous spinules and in the shape of gonopods, it can be distinguished from the latter species by the arista; in the new species, arista with 1 (mostly) or 2 (occasionally) dorsal branches, and several spinules at tip (Fig. 7). (In *P. longipennis*: arista with 2–3 dorsal branches basally and 3 spinules at tip; as in Chen *et al.*, 2005, fig. 4A).

**MEASUREMENTS:** BL = 3.33 mm in holotype (range in 5♂ paratypes: 2.93–3.33), THL = 1.67 mm (1.27–1.67), WL = 2.53 mm (2.80–3.33), WW = 1.00 mm (0.93–1.33), arb = 1/0 (1–2/0), adf = 0.86 (0.50–1.00), flw = 1.71 (1.50–1.75), FW/HW = 0.38 (0.32–0.50), ch/o = 0.12 (0.08–0.14), prorb = 1.22 (1.14–1.83), rcorb = 0.33 (0.29–0.67), vb = 0.60 (0.40–0.80), dcl = 0.44

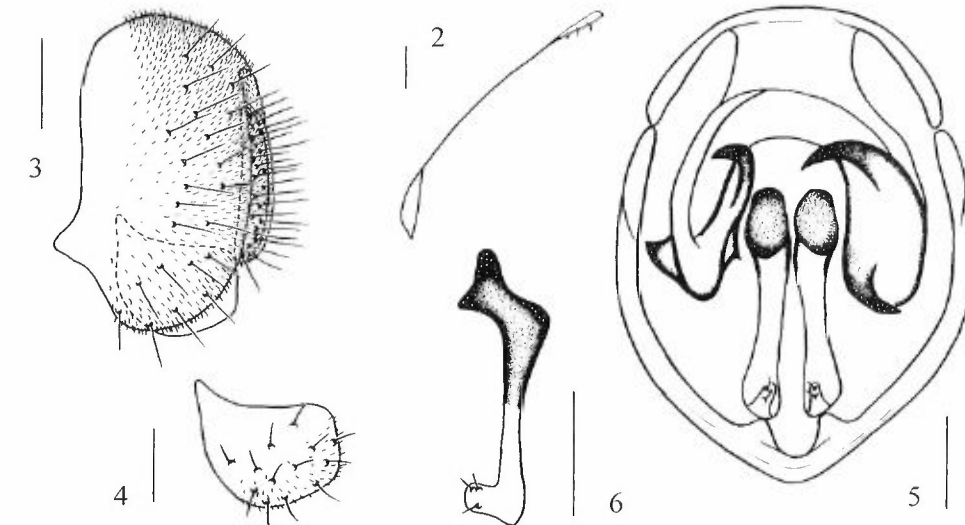


Fig. 2–6. *Phortica* (*Ashima*) *haba* An & Chen, sp. nov., male. 2. Arista; 3. Epandrium and cercus (lateral view); 4. Surstylus (ventral view); 5. Hypandrium, parameres, gonopods and aedeagus (ventral view); 6. Paramere (lateral view). Scale bars = 0.1 mm.

(0.29–0.76), presctl = 0.50 (0.63–0.94), sctl = 1.05 (1.00–1.12), sterno = 0.89 (0.88–1.33), orbito = 1.33 (1.25–2.17), dcp = 0.31 (0.26–0.33), sctlp = 1.33 (1.00–1.33), C = 2.00 (2.05–2.55), 4c = 1.77 (1.43–2.10), 4v = 3.08 (2.71–3.73), 5x = 1.00 (0.70–0.89), ac = 3.83 (2.86–3.50), M = 0.69 (0.50–0.83), C3F = 0.70 (0.67–0.80).

## Description

Frons black, with thick and dense interfrontal setae. Pedicel black; first flagellomere brownish. Face dark brown, with white patches on lower corners. Clypeus medially white, laterally black. Thorax black anteriorly, orange brown posteriorly. Scutellum nearly black. Legs yellow; fifth tarsomere of foreleg with long seta apically. Abdominal tergites third and fourth with medially interrupted black bands on posterior margins; tergites fifth and sixth nearly entirely black. Atemites third to fifth distinctly broadened, shorter than wide, with several long setae laterally. Male terminalia: Surstylus with several setae and *c.* 12 prenisetae on ventral margin to inner surface (Fig. 9). Paramere weakly sclerotized and smooth apically, with 4 sensilla basally (Figs 10, 11). Aedeagus median rod slender (Fig. 10). Vertical process of gonopods asymmetrical, strongly sclerotized on anterior margin, distally and basally each with 2 processes right side (Fig. 10).

*Phortica* (*Ashima*) *qingsongi* An & Chen, sp. nov.  
(Figs 12–15)

**HOLOTYPE:** SCAU, DIP 124121, ♂ (23.08.2013).

**TYPE LOCALITY:** CHINA: Baihualing, Baoshan, Yunnan, 1400 m a.s.l., QS Gao.

**PARATYPE:** SCAU, DIP 124122, 1♂ (23.08.2013), same data as holotype.

**ETYMOLOGY:** Patronym of the collector, Mr Qingsong Gao (SCAU).

**DIAGNOSIS:** Similar to *P. afofoliata* and *P. eseta* in the shape of male arista, differs in the shape of male genitalia: Vertical process of gonopods asymmetrically sclerotized, with 2 projections dorsally (Figs 14, 15); aedeagal median rod sclerotized, with 2 small projections and 1 separate, furcate sclerite (Figs 14, 15).

**MEASUREMENTS:** BL = 3.40 mm (3.20 in 1♂ paratype), THL = 1.47 mm (1.27), WL = 2.93 mm (2.67), WW = 1.13 mm (1.07), arb = 3/2 (3/2), avd = 0.50 (0.60), adf = 1.00 (1.00), flw = 1.75 (1.40), FW/HW = 0.45 (0.50), ch/o = 0.08 (0.11), prorb = 1.22 (1.13), rcorb = 0.83 (0.63), vb = 0.80 (0.60), dcl = 0.40 (0.40), presctl = 0.40 (0.40), sctl = 1.00 (1.00), sterno = 0.95 (0.94), orbito = 1.55 (1.50), dcp = 0.26 (0.25), sctlp = 1.15 (1.00), C = 1.08 (1.30), 4c = 1.54 (1.75), 4v = 3.08 (3.25), 5x = 1.00 (1.11), ac = 5.00 (3.00), M = 0.69 (0.83), C3F = 0.50 (0.71).

## Description

Frons black above, brownish yellow below, medially with thick, dense, minute interfrontal setulae. Male arista plumose, apically not expanded. Pedicel and first flagellomere brownish yellow. Face brown, with yellowish white patches on lower corners. Clypeus medially white,



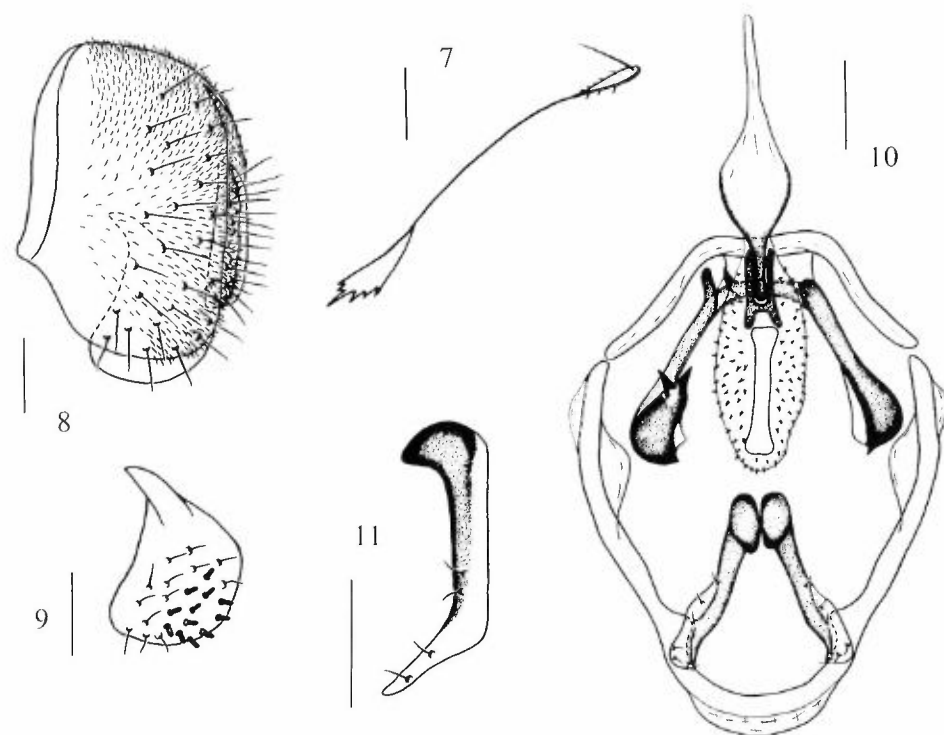


Fig. 7–11. *Phortica (Ashima) montipagana* An & Chen, sp. nov., male. 7. Arista; 8. Epandrium and cercus (lateral view); 9. Surstylus (ventral view); 10. Hypandrium, parameres, gonopods and aedeagus (ventral view); 11. Paramere (lateral view). Scale bars = 0.1 mm.

laterally black. Thorax black anteriorly and medially, orange brown posteriorly and laterally. Scutellum orange brown. Legs yellow, with dark spots submedially on all femora; all tibiae with 2 dark rings; fifth tarsomere of

foreleg without long seta apically. Abdominal tergites third to fifth yellow, with broad, brownish-black posterior bands interrupted medially. Sternites third to fifth longer than wide, with a few of long setae laterally. Male

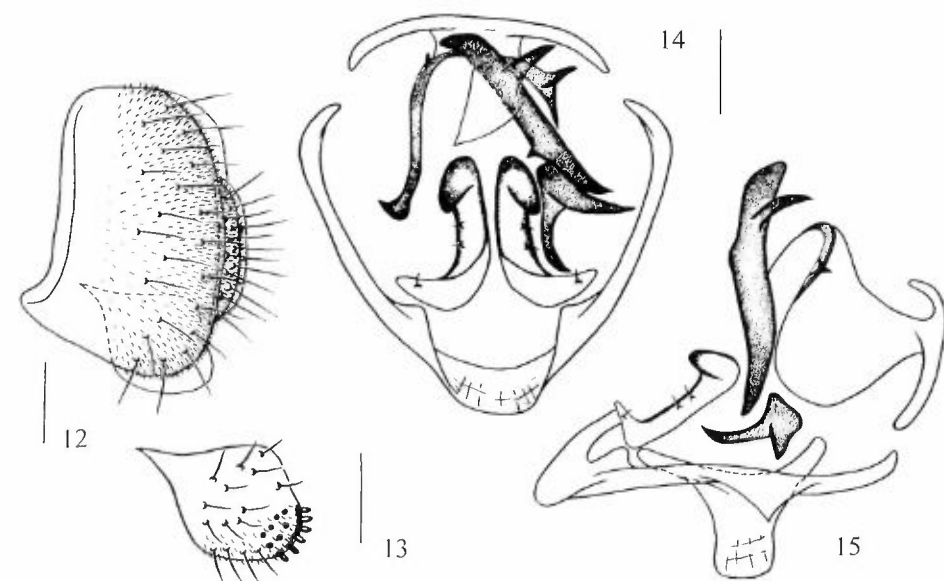


Fig. 12–15. *Phortica (Ashima) qingsongi* An & Chen, sp. nov., male. 12. Epandrium and cercus (lateral view); 13. Surstylus (ventral view); 14, 15. Hypandrium, parameres, gonopods and aedeagus (ventral and lateral views). Scale bars = 0.1 mm.

terminalia: Epandrium mid-dorsally constricted, laterally broad, with pubescence and dense setae (Fig. 12). Surstylus with pubescence, numerous setae, and prensisetae on ventral margin to inner surface (Fig. 13). Paramere strongly sclerotized, knobbed apically, and with 3 sensilla basally to submedially (Figs 14, 15).

## Discussion

Although some diagnostic characters were recognized, the morphological differences could not delimit the cryptic species, in particular the three new species *P. haba*, *P. montipagana* and *P. qingsongi* in the present study. In general, species with extremely similar morphology could be considered as the cryptic ones, even exhibiting obvious genetic divergence with others. Thus, it is difficult to distinguish the species from each other. Molecular data could be a choice to circumvent this problem. *Phortica haba* is similar to *P. brachychaeta* and *P. nudiarista* in the arista lacking branches (Fig. 2) and the shape of paramere (Fig. 6). *Phortica montipagana* is close to *P. longipennis* in the morphological characters, e.g. aedeagal outer membrane with numerous spinules and the shape of gonopods. This suggests that the delineations of *P. haba* and *P. montipagana* have been obscure. However, the minimum interspecific genetic distance for *P. haba* and *P. montipagana* with others was 2.1% and 3.0%, respectively. These minimum interspecific genetic distances were comparable with others calculated in the present study (Table 2), indicating the independence of these potential cryptic species, *P. haba* and *P. montipagana*, versus others. This is also consistent with the result of the phylogenetic analysis based on maximum likelihood or Bayesian criterion (Fig. 1) representing a good example about the benefits from using DNA barcoding to provide clear delimitation of cryptic species. For the other new species, *P. qingsongi*, the minimum interspecific genetic distance is only 0.5% against *P. afoliolata* showing remarkably weak support, in agreement with the phylogenetic analysis (Fig. 1). The geographic barrier between *P. qingsongi* and *P. afoliolata* is a potential reason for the speciation. Based on the previous phylogenetic analysis (Cao *et al.*, 2011), *P. afoliolata* could be split after the formation of Hainan Island with short speciation time, resulting in the less genetic distance of the mitochondrial gene *COI*. Thus, *P. qingsongi* was still designated as new species, characterized by morphological differences described above. Results from the present study confirm that both morphological and molecular data are complementary to delineate new species.

The phylogenetic relationships within the subgenus *Ashima* herein look different from the previous study (Cao *et al.*, 2011). The basal position of Clade I was proposed (Cao *et al.*, 2011), while Clade I has been embedded into the various clades of the subgenus *Ashima* in the

present study (Fig. 1). This is possibly attributed to the less phylogenetic information from the sole *COI* gene. Another possibility could be that some new species incorporated in the present study affected the stability of the topology. Thus the phylogenetic relationships from the previous study were temporally reconstructed. However, weak support for most internal nodes in the present phylogenetic tree suggested more concatenated genes from possibly more specimens should be necessary in further studies. The lineage diversification within *Ashima* was proposed to occur in South China and adjacent areas on the basis of the current distribution patterns. In agreement with the previous studies, the diversification of the subgenus *Ashima* was possibly triggered in South China since mid-Miocene, and this could be associated with the uplift of the Himalaya-Tibetan Plateau in the later Miocene (Cao *et al.*, 2011). In addition, this is also in accordance with the habitat distribution pattern of the subgenus *Ashima* in South China (Table 1). The unprecedented diversity of the subgenus *Ashima* was observed in the mountain forests of South China, this diversity being much higher than in the plain areas of the same region. As mentioned previously and indicated by the current southern China–South-East Asia distribution of some species within the subgenus *Ashima* (for instance, *P. foliiseta*, *P. spinosa* and *P. tanabei*), dispersals from southern China to South-East Asia may have occurred subsequent to the speciation of these species. Presumably, these dispersals were facilitated by the climate changes associated with the uplift of the Himalaya–Tibetan Plateau. In addition, several intriguing observations about distributions within the subgenus *Ashima* were addressed in the present study. For instance, *Ashima* species from Hainan are close to those from Yunnan, but not the adjacent area, Guangdong of China. One example concerns *P. qingsongi* from Yunnan and *P. afoliolata* from Hainan. The similar habitats of Hainan and Yunnan could be the main driving force to shape this distribution pattern discussed above. It is in accordance with the observation of higher species diversity in mountain forest than in the plain. South-western China is featured with high mountains and plateaus, but rarely in relatively low altitude areas like those in south-eastern China. Taken together, the biased distribution pattern of the subgenus *Ashima* suggests the potentially specific adaptation of *Ashima* to high plateau environments.

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## References

- Akaike, H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, *19*, 716–723. doi: 10.1109/TAC.1974.1100705.
- Anisimova, M. & Gascuel, O. (2006) Approximate Likelihood-Ratio Test for Branches: A Fast, Accurate, and Powerful Alternative. *Systematic Biology*, *55*, 539–552. doi: 10.1080/10635150600755453.
- Cao, H. L., Wang, X. L., Gao, J. J., Prigent, S. R., Watabe, H., Zhang, Y. P., & Chen, H. W. (2011). Phylogeny of the African and Asian *Phortica* (Drosophilidae) deduced from nuclear and mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, *61*, 677–685. doi: 10.1016/j.ympev.2011.08.002.
- Chen, H. W., Gao, J. J., & Wen, S. Y. (2005). Species diversity of the genus *Phortica* Schiner in Yunnan, China, with descriptions of nine new species (Diptera, Drosophilidae). *Journal of Natural History*, *39*, 3951–3978. doi: 10.1080/00222930500533534.
- Chen, H. W. & Máca, J. (2012). Ten new species of the genus *Phortica* from the Afrotropical and Oriental regions (Diptera: Drosophilidae). *Zootaxa*, *3478*, 493–509.
- Chen, H. W., & Toda, M. J. (2001). A revision of the Asian and European species in the subgenus *Amiota* Loew (Diptera, Drosophilidae) and the establishment of species-groups based on phylogenetic analysis. *Journal of Natural History*, *35*, 1517–1563. doi: 10.1080/002229301317067665.
- Cheng, Y., Gao, J. J., Watabe, H., & Chen, H. W. (2008). Revision of the genus *Phortica* Schiner, 1862 in China (Diptera: Drosophilidae). *Zoological Studies*, *47*, 614–632.
- Drummond, A. J., & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BioMed Central Evolutionary Biology*, *7*, 214. doi: 10.1186/1471-2148-7-214.
- Duda, O. (1923). Die orientalischen und australischen Drosophiliden-Arten (Dipteren) des Ungarischen National Museums zu Budapest. *Annales Historico-Naturales Musei Nationalis Hungarici*, *20*, 24–59.
- Folmer, O., Black, M., Hoh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–299.
- Gao, Q. S. & Chen, H. W. (2014). The genera *Luzonimyia* and *Pararhinoleucophenga* from China (Diptera: Drosophilidae), with DNA barcoding information. *Zootaxa*, *3852*, 294–300. doi: 10.11646/zootaxa.3852.2.8.
- Guindon, S., & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, *52*, 696–704. doi: 10.1080/10635150390235520.
- He, X.F., Gao, J.J., Cao, H.Z., Zhang, X.L., Chen, H.W. (2009) Taxonomy and molecular phylogeny of the *Phortica hani* species complex (Diptera: Drosophilidae). *Zoological Journal of the Linnean Society*, *157*, 359–372. doi: 10.1111/j.1096-3642.2009.00516.x.
- Huang, J., Li, T., & Chen, H. W. (2013a). The genus *Leucophenga* (Diptera, Drosophilidae), part III: the *interrupta* species group from the Oriental region, with morphological and molecular evidence. *Zootaxa*, *3750*, 587–600. doi: 10.11646/zootaxa.3750.5.9.
- Huang, J., Li, T., & Chen, H. W. (2014). The genus *Leucophenga* (Diptera, Drosophilidae), part IV: the *ornata* species group from the East Asia, with morphological and molecular evidence (II). *Zootaxa* (in press).
- Huang, J., Li, T., Gao, J. J., & Chen, H. W. (2013b). The genus *Leucophenga* (Diptera, Drosophilidae), part II: the *ornata* species group from East Asia, with morphological and molecular evidence (I). *Zootaxa*, *3701*, 101–147. doi: 10.11646/zootaxa.3701.2.1.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*, 111–120. doi: 10.1007/BF01731581.
- Lanave, C., Preparata, G., Saccone, C., & Serio, G. (1984). A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution*, *20*, 86–93. doi: 10.1007/BF02101990.
- Li, N. N., Toda, M. J., Fu, Z., Chen, J. M., Li, S. H., & Gao, J. J. (2014). Taxonomy of the *Colocasiomyia gigantea* species group (Diptera, Drosophilidae), with descriptions of four new species from Yunnan, China. *Zookeys*, *406*, 41–64. doi: 10.3897/zookeys.406.7176.
- Máca, J. (2003). Taxonomic notes on the genera previously classified in the genus *Amiota* Loew (Diptera, Drosophilidae, Steganinae). *Acta Universitatis Carolinae Biologica*, *47*, 247–274.
- Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology*, *7*, 16. doi: 10.1186/17429994716.
- Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, *25*, 1253–1256. doi: 10.1093/molbev/msn083.
- Rach, J., DeSalle, R., Sarkar, I. N., Schierwater, B., & Hadrys, H. (2008). Character-based DNA barcoding allows discrimination of genera, species and populations in Odonata. *Proceedings of the Royal Society of London Series B – Biological Sciences*, *275*, 237–247. doi: 10.1098/rspb.2007.1290.
- Rodriguez, F., Oliver, J. L., Marin, A., & Medina, J. R. (1990). The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology*, *142*, 485–501. doi: 10.1016/S00225193(05)801043.
- Schiner, I. R. (1862). Vorläufiger Commentar zum dipterologischen Theile der “Fauna Austriaca”. *Wiener Entomologische Monatschrift*, *6*, 428–436.
- Su, Y. R., Lu, J. M., & Chen, H. W. (2013). The genus *Leucophenga* (Diptera, Drosophilidae), part I: the *abbreviata* species group from the Oriental region with morphological and molecular evidence. *Zootaxa*, *3637*, 361–373. doi: 10.11646/zootaxa.3637.3.8.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*, 2731–2739. doi: 10.1093/molbev/msr121.
- Wang, B. C., Park, J., Watabe, H., Gao, J. J., Xiangyu, J. G., Aotsuka, T., Otsuka, T., ... Zhang, Y. P. (2006). Molecular phylogeny of the *Drosophila virilis* section (Diptera: Drosophilidae) based on mitochondrial and nuclear sequences. *Molecular Phylogenetics and Evolution*, *40*, 484–500. doi: 10.1016/j.ympev.2006.03.026.
- Yang, Z. F., Landry, J. F., Handfield, L., Zhang, Y. L., Solis, M. A., Handfield, D., Scholtens, B. G., ... Hebert, P. D. N. (2012). DNA barcoding and morphology reveal three cryptic species of *Anania* (Lepidoptera: Crambidae: Pyraustinae) in North America, all distinct from their European counterpart. *Systematic Entomology*, *37*, 686–705. doi: 10.1111/j.1365-3113.2012.00637.x.
- Yassin, A., Capy, P., Madi-Ravazzi, L., Ogereau, D., & David, J. R. (2008). DNA barcode discovers two cryptic species and two geographical radiations in the invasive drosophilid *Zaprionus indianus*. *Molecular Ecology Resources*, *8*, 491–501. doi: 10.1111/j.1471-8286.2007.02020.x
- Yassin, A., Markow, T. A., Narechania, A., O’Grad, P. M., & DeSalle, R. (2010). The genus *Drosophila* as a model for testing tree- and character-based methods of species identification using DNA barcoding. *Molecular Phylogenetics and Evolution*, *57*, 509–517. doi: 10.1016/j.ympev.2010.08.020.
- Zhang, W. X., & Toda, M. J. (1992). A new species-subgroup of the *Drosophila immigrans* species-group (Diptera, Drosophilidae), with description of two new species from China and revision of taxonomic terminology. *Japanese Journal of Entomology*, *60*, 839–850.
- Zhang, Y., Tsaur, S. C., & Chen, H. W. (2014). Survey of the genus *Stegana* Meigen (Diptera, Drosophilidae) from Taiwan, with DNA barcodes and descriptions of three new species. *Zoological Studies*, *53*, 1–15. doi: 10.1186/1810-522X-53-2.
- Zhang, Y., Xu, M. F., & Chen, H. W. (2012). Revision of the subgenus *Stegana* (*Orthostegana*) from eastern Asia (Diptera, Drosophilidae). *Entomotaxonomia*, *34*, 361–374.
- Zhao, F., Gao, J. J., & Chen, H. W. (2009). Taxonomy and molecular phylogeny of the Asian *Paraleucophenga* Hendel (Diptera: Drosophilidae). *Zoological Journal of the Linnean Society*, *155*, 616–629. doi: 10.1111/j.1096-3642.2008.00450.x.
- Zou, S., Li Q., Kong, L., Yu, H., & Zheng, X. (2011). Comparing the usefulness of distance, monophyly and character-based DNA barcoding methods in species identification: a case study of Neogastropoda. *Public Library of Science ONE*, *6*, e26619. doi: 10.1371/journal.pone.0026619.

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