

A new record of *Iranihindia martellata* (Senior-White, 1924) (Diptera: Sarcophagidae) from peninsular Malaysia and female identification using both morphology and DNA-based approaches

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Abstract. *Iranihindia martellata* (Senior-White, 1924) is recorded from peninsular Malaysia for the first time. Male and female specimens in the recent collections of forensically important sarcophagid flies were examined and identified based on morphology and DNA sequencing analysis. Male genitalia offer unambiguous species identification characteristics in the traditional taxonomy of flesh flies but the female flies are very similar to one another in general morphology. Female of *I. martellata* was determined by DNA sequencing (*COI* and *COII*) and PCR-RFLP (*COI*) analysis. Identified females were carefully examined and compared with the morphologically similar species, *Liopygia ruficornis* (Fabricius, 1794). Female genitalia are re-described and illustrated in this paper.

INTRODUCTION

Iranihindia martellata (Senior-White, 1924) is recorded for the first time from peninsular Malaysia in recent surveys of forensically important sarcophagid flies. The genus *Iranihindia* Rohdendorf, 1961 is documented to be widely distributed in the Oriental and Palaearctic regions such as Nepal, Sri Lanka, India and Iran (Pape, 1996; Nandi, 2002). Type locality of *I. martellata* (Senior-White) is Niroddumunai, Sri Lanka (Pape, 1996). The genus *Iranihindia* Rohdendorf was established under the subtribe Harpagophallina Rohdendorf in the subfamily of Sarcophaginae Macquart (Essig, 1947; Pape, 1996; Nandi, 2002).

There are nine species in the genus *Iranihindia* Rohdendorf, namely *Iranihindia futilis* (Senior-White, 1924), *Iranihindia indica* Nandi, 1979, *Iranihindia jamesi* Nandi, 1979, *Iranihindia martellatoides* (Baranov, 1931), *Iranihindia nepalensis* Kano & Shinonaga, 1969, *Iranihindia persica* Rohdendorf, 1961, *Iranihindia spinosa* Li, Ye & Liu, 1985, *Iranihindia zaitzevi* Verves, 1989 and *I. martellata* (Senior-White). However, the distributions for all these species are mainly in India, Nepal and Sri Lanka, to a lesser extent in Iran, China, Myanmar and Pakistan (Kano & Shinonaga, 1969; Fan 1992; Pape, 1996; Nandi, 2002). Out of the nine species, six can be found in India where *Iranihindia* Rohdendorf is most abundant (Nandi, 2002).

Morphology-based identification for most female specimens of sarcophagid flies is complicated or sometimes impossible even for the experienced entomologist due to high similarities of their anatomy (Byrd & Castner, 2001). DNA-based identification provides a useful alternative to overcome the problem of identification for female specimens, by comparison with DNA sequences of the corresponding male specimens. Confirmation of the female specimen's identity (using DNA-based approaches) would then allow the defining morphological characters of female species to be re-examined, analyzed and recorded for future reference.

In this paper, the identity of male *I. martellata* was confirmed through examination of characteristic features of the male genitalia. However, our observations of the morphological characteristics of the female genitalia of this species do not concur with those described by Nandi (2002). The female genitalia are thus re-described herein, based on female specimens of which the identity recognition have been supported by data from DNA sequencing.

MATERIALS AND METHODS

Collection of fly specimens

Sample collections of fly survey (Diptera: Sarcophagidae) were carried out throughout peninsular Malaysia during the years of 2006-2008. Live flies were collected in different geographical topologies using two-day-old spoiled beef as bait and the sweeping net method was used to capture the flies. The localities of *I. martellata* specimens collected are described below.

Specimens examined and used for analysis

MALAYSIA: Malaya, Kuala Lumpur, University of Malaya, Genetics, garden, 1 ♀... (4.viii.2006), 2 ♂... (26.viii.2006), S.H. Tan; Kuala Lumpur, University of Malaya, Rimba Ilmu botanical garden, 1 ♂... (19.iii.2007), 4 ♀... (2.x.2007), 1 ♂ & 1 ♀...

(17.x.2007), 2 ♀... (24/25.xi.2007), 1 ♀... (11.xii.2007), 1 ♀... (11.iii.2008), S.H. Tan; Kuala Lumpur, University of Malaya, Varsity Lake, 2 ♂... (15.x.2007), 1 ♂... (11.xii.2007), S.H. Tan; Kuala Lumpur, University of Malaya, 7th Residential College, field, 1 ♂... (11.viii.2007), J.C.M. Sau; Kuala Lumpur, Old Klang Road, OBD Condominium, bushes of residential area, 1 ♂ & 1 ♀... (11.iix.2007), S.H. Tan; Kuala Lumpur, Old Klang Road, Taman Abadi Indah, field, 2 ♂... (11.iiv.2007), S.H. Tan; Johor State, Johor Bahru District, Istana Garden, grassland, 1 ♂ & 1 ♀... (31.viii.2007), J.C.M. Sau; Selangor State, Petaling District, Subang Jaya USJ6, residential area, garden, 1 ♂... (18.xi.2007), F.D. Khaidizar; Negeri Sembilan State, Port Dickson District, Port Dickson, sandy beach, 2 ♂... (8.v.2008), H. Kurahashi and S.H. Tan.

Morphology-based identification

The flies were killed with ethyl acetate and pinned. The male and female genitalia were cut and immersed in 10% KOH overnight. Then the genitalia were dissected in water and the tracheae and other tissues were carefully removed. The genitalia and 5th sternite were examined under microscope and illustrated. Finally, the genitalia was either kept with glycerol in glass vials with cork stopper or mounted on a piece of card with Neo-Shigalal[®] mounting media (Tokyo, Japan) for long term storage. The voucher specimens were kept in the Division of Genetics and Molecular Biology, University of Malaya for future reference.

DNA-based identification

Male and female fly specimens were subjected to DNA extraction using two legs from each individual of *I. martellata*. Preferably, only legs from one side of the body were used so that characters useful for species identification can be preserved. Total genomic DNA was extracted using QIAamp[®] Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

The *cytochrome oxidase subunit I* and *II* (*COI* and *COII*) genes in the *I. martellata* mitochondrial genome were amplified

using polymerase chain reaction (PCR) and then subjected to DNA sequencing. PCR was carried out in 50µL reactions, with the final concentrations of 100ng template DNA, 1 unit of *Taq* polymerase, 1× PCR reaction buffer, 2mM MgCl₂ (Biotools, Spain), 200µM of each dNTP (Fermentas, USA) and 0.4µM of each forward and reverse primer (Bio Basic, Canada). Amplification reactions were performed in the MJ Research PTC-200 thermal cycler (MJ Research, USA). Two pairs of primers used in this study were as previously described (Sperling *et al.*, 1994; Wells & Sperling, 2001; Schroeder *et al.*, 2003). PCR parameters include an initial denaturation step of 94°C of 5 minutes, followed by 35 cycles of 94°C for 1 minute, 45°C for 1 minute 30 seconds, 72°C for 2 minutes, followed by a final elongation step for 72°C for 5 minutes.

PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) or QIAquick® Gel Extraction Kit (Qiagen). The QIAquick® Gel Extraction Kit was used when the PCR product yields unspecific amplification fragment. Purified PCR products were then cloned into the pGEM®-T Easy vector system (Promega, USA) to facilitate DNA sequencing procedures. Sequencing was performed using ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, USA) according to the manufacturer's recommendations. All samples were sequenced for both forward and reverse DNA strands using the universal M13 forward and reverse primers. Electrophoresis and detection of the sequencing reaction products was carried out in the capillary electrophoresis system ABI PRISM 3130xl Genetic Analyzer with a capillary length of 80 cm. The DNA sequence reads were edited manually using the Chromas software (v2.32) to eliminate discrepancies between forward and reverse sequences.

We also assessed the usefulness of a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to distinguish *I. martellata* and one of its similar species, *Liopygia*

ruficornis (Fabricius, 1794). For the PCR-RFLP analysis, the 1380bp *COI* PCR product was digested with restriction endonuclease *SspI* (New England Biolabs, USA) according to the manufacturer's recommendation. Then, the digested sample was resolved by electrophoresis in 2% TBE agarose gel, stained with ethidium bromide and viewed under UV-illumination. The restriction patterns were photographed and analyzed.

RESULTS AND DISCUSSION

Our study presents the first record of *I. martellata* in peninsular Malaysia. *Iranihindia martellata* is a common species and can be collected from bushes and flowering plants in India (Nandi, 2002). Morphological characteristics of *I. martellata* are very similar to those of *I. martellatoides*. Nandi (2002) later stated that *I. martellata* has unique shape of harpes, which is plough-like in structure. Nandi (2002) also distinguished *I. martellata* from four other species of *Iranihindia* in India (*I. futilis*, *I. jamesi*, *I. martellatoides* and *I. indica*) solely by using the character of third abdominal tergite with or without weak median marginal bristles.

In our study, this species was collected mostly from the bushes and some flowering plants. Our attempts to establish a colony in the laboratory were not successful as larvae deposited on meat failed to survive. Perhaps this is expected because our observation on the larvae behaviour, as well as in previous reports indicated that *I. martellata* is a scatophagous (feeding on excrement) species. This species has been shown to breed on rabbit carcass and human excrement (Senior-White *et al.*, 1940; Nandi, 2002).

Upon closer examination of their morphology, the female genital structures of this fly were notably different from those illustrated in Nandi (2002). The apparent lack of detailed illustration and description of the female genital structures (see figure 873; Nandi, 2002) made comparison

difficult, and could contribute towards discrepancies noted in our study. For general morphological identification, a photograph of male *I. martellata* is shown in Fig. 1. For clarity and to facilitate re-description, an illustration of the female genitalia of *I. martellata* is shown in Fig. 2. Female postabdomen comprises a genitalia or ovipositor. The length is short, its type is the so-called *Sarcophaga*-type which is commonly found among larviparous species of Sarcophagine flesh flies. Tergite 5 (T5) of the last preabdominal segment, normal shaped, without remarkable structure, largely clothed in black hairs, with row of strong marginal bristles; tergite 6 (T6) of complete plate-like sclerite, clothed in black hairs on posterior 1/3 of lateral side, with two separated rows of marginal bristles on left and right lateral sides; tergite 7 (T7) absent; tergite 8 (T8) reduced to small sclerite, and present on medially, bare, without bristle; sternite 5 (S5) of normal elongate plate, clothed in black hairs on anterior 3/4, with row of marginal bristles; sternite 6 (S6) of normal broad plate, clothed in black hairs on anterior 3/4, with row of marginal bristles; sternite 7 (S7) of large and broad plate with round lateral margins, anterior margin amalgamated with posterior margin of sternite 8 (S8), clothed in black hairs on anterior 1/2, with two lateral rows of 3-4 marginal bristles; sternite 8 (S8) of small, but broad plate, bare on sclerite, membranous portion bearing minute hairs along anterior margin of genital pore, without bristle; supraanal plate (SAP) absent; subanal plate (SBAP) developed; cercus (ce) normal in size, clothed with black hairs except for disc bare, with several fine long bristles along inner margin and one on outer margin; spermatheca globulous, without distinct sculpture pattern; there is a star-shaped secondary pigmented plate-like area present between tergite 6 and tergite 8 in some of the female specimens.

For DNA-based identification analysis, the mitochondrial DNA *COI* and *COII* sequences obtained were 2305bp in length for all the individuals studied. Two

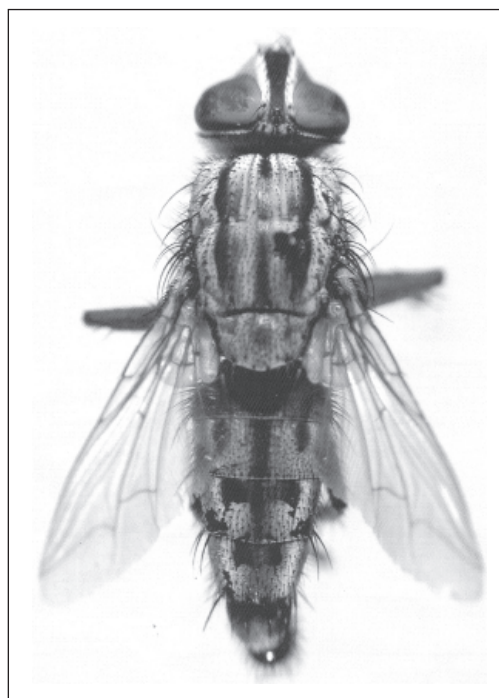


Figure 1. Dorsal view of male *I. martellata*, new record from peninsular Malaysia

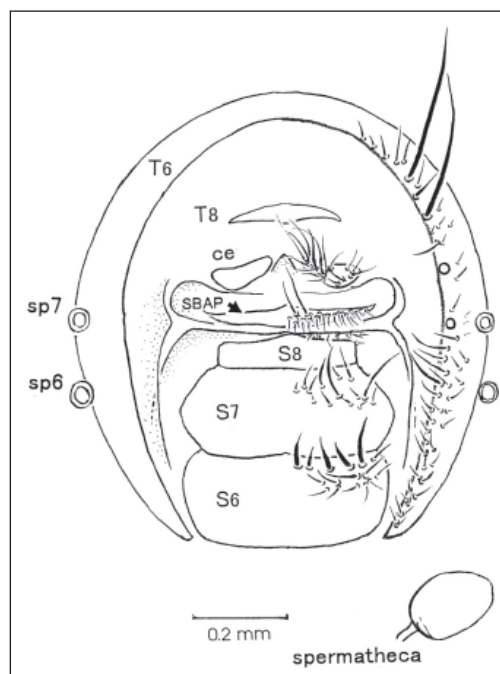


Figure 2. Female genitalia of *I. martellata*, caudal view: spiracle 6 (sp6), spiracle 7 (sp7), tergite 6 (T6); tergite 7 (T7), sternite 6 (S6), sternite 7 (S7), sternite 8 (S8), subanal plate (SBAP), cercus (ce) and spermatheca

sequences that represent variants were deposited into GenBank with accession numbers of FJ440843 and FJ440844. These regions included the cytochrome oxidase subunit I and II genes (*COI* and *COII*), the tRNA leucine gene and a spacer region. This mtDNA region was observed to have a strong AT bias (average 69%), which is typical of insect mitochondrial DNA (Crozier & Crozier, 1993). We also included one morphologically similar species which is easily obtained from Malaysia, *Liopygia ruficornis* in the DNA-based analysis. Intra-specific variation for *I. martellata* showed a maximum of 0.6% whereas the interspecific variation between *I. martellata* and *L. ruficornis* (GenBank accession number: EF405940) is

approximately 9.1%. Similar results were reported by Wells & Sperling (1999) for *Chrysomya albiceps* (Wiedemann) and *Chrysomya rufifacies* (Macquart) for which their intraspecific variations are less than 1%, and by Wells *et al.* (2001) where interspecific variations of sarcophagid species are shown to be more than 3%. The enormous interspecific variation between *I. martellata* and *L. ruficornis* facilitated the unambiguous identification of these two species.

From the PCR-RFLP analysis, *I. martellata* showed a distinct and unique restriction pattern compared to its closely related species, *L. ruficornis* (Fig. 3). Both species are commonly present in the same collection site, and show high similarities

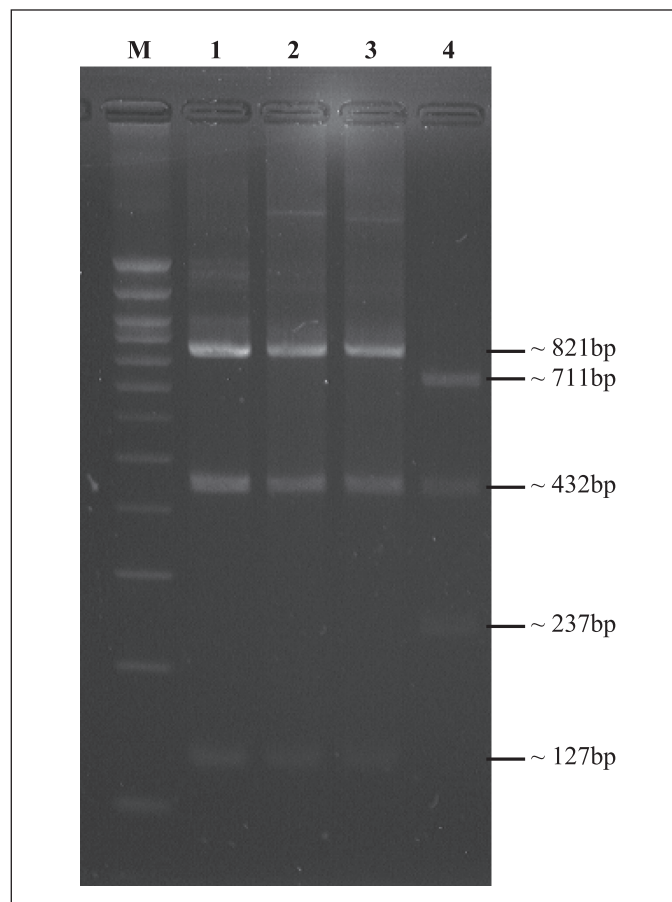


Figure 3. PCR-RFLP assay of 1380bp *COI* region digested with *SspI* (NEB, USA). Lane 1 is female specimen of *I. martellata*, Lanes 2 and 3 are male specimens of *I. martellata* while Lane 4 is *L. ruficornis*. M is the 100bp ladder (Seegene, Korea)

in morphology and behaviour. They are synanthropic species and possess yellow to orange colour antennal segments and palpi. Male and female specimens of *I. martellata* showed consistent restriction fragments of 821bp, 432bp and 127bp, while *L. ruficornis* showed restriction fragments of 711bp, 432bp and 237bp. These results suggest that PCR-RFLP assay should be useful to distinguish these two species.

In this study, we utilised variations in both morphological characters and DNA sequence for the identification of ambiguous flesh fly specimens. While the characteristic morphology of the male genitalia has been accepted as a definitive taxonomical point of reference for male flies, identification of female flies is notoriously difficult. Based on the presence of apomorphic substitutions present in the mtDNA sequences in identified males, reliable identification of females can be achieved by inference of sequence similarities. Confirmation of the female identity has also allowed the morphology of the female genitalia to be examined and compared to available descriptions, and perhaps re-described when warranted. The collaboration of morphology-based and DNA-based identifications has indeed proven useful and should be considered in resolving taxonomical conflicts.

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