Identification of forensically important sarcophagidae (Diptera) by DNA-based method coupled with morphological characteristics

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Abstract: The Sarcophagidae, besides the Calliphoridae, are considered as forensically important insects for investigating postmortem interval (PMI), however, often be neglected. Using decomposing pig bodies with various postmortem intervals, we identified 47 sarcophagid flies from hundreds of fly specimens collected at several Taiwan areas. Species diversity of the sarcophagid flies was further studied based on both morphological identification and DNA analysis at the mitochondrial oxidase subunit I (COI) gene.

Seven species, Liopygia crassipalpis, Parasarcophaga misera, Boettcherisca pergrina, B. formosensis, and Seniorwhitea princeps, were initially identified by microscopic observation. Nineteen of the 47 sarcophagid flies were categorized to be P. dux. The COI DNA sequence variations within intra-species were less than 0.8% for L. crassipalpis, P. misera, L. ruficornis, S. princeps, B. formosensis, 1.2% for B. pergrina, and 1.8% for P. dux. Our results were consistent to the literature's finding; i.e., DNA variations in intra-species of P. dux were higher than those of other sarcophagid species.

Neighbor-Joining tree results revealed that there were three subgroups within the population of P. dux, and the COI DNA sequence of the three was found to be highly variable, but was of high similarity within same subgroup. Morphologically, the 19 P. dux specimens had high similarity, though with minor difference among the three subgroups. The difference was found to locate at pregonite and the color of 2nd genital tergite. The finding of the relationship between DNA sequence variations and morphological features among the intra-species of P. dux was first reported.

Key Words: forensic entomology, sarcophagidae, mitochondrial DNA, intra-species variation.

The major use of forensic entomology is estimating the time of death or the post mortem interval (PMI) of a human corpse [1-3]. This is done by identifying the species and the age of the insects on a corpse. In practice, when a body is found, the investigator would record the time the insects developing on the corpse to reach the oldest stage. In Taiwan, the blow flies (Diptera: Calliphoridae) are usually the first insects to colonize a body after death.

Accordingly, we [4] and others [5] have identified and characterized this forensically important blow flies’ species in Asia. Among the useful insects for estimating the PMI, sarcophagid flies (flesh flies) are also considered useful, because the Sarcophagidae colonize a corpse in an early stage and the size of their larvae is big, easily to be picked up for analyses. The forensic importance and potentiality of Sarcophagidae has been emphasized in literature [6-8]. Specifically, the sarcophagids (viviparous) can provide more accurate PMI estimation over that of the

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calliphorids (oviparous), majorly due to the feature of a direct correlation between the developed immature larva stage and the corpse’s decomposition level [9]. Nevertheless, compared to the Calliphoridae, application of the Sarcophagidae on forensic practice so far is quite restricted, mainly due to two reasons: too many sarcophagid species and the difficulty of species identification of larval stage based on anatomy [6]. The sarcophagid flies are ubiquitous in human environment and are known to consist of thousands species with diverse habits, for instance, live mammalian tissue, feces, decomposing body, and agricultural spots [6]. Although only a part of sarcophagid flies are classified to carrion breeders, they still comprise many species. Serving as insect evidence, once misidentifying the sarcophagid specimen, it would lead to an inaccurate PMI estimation. Moreover, the Sarcophagidae could be locus-specific, having distinct environmental and faunal features. Accordingly, the knowledge and establishment of local sarcophagid fauna would be indispensable, because the data from other regions are not appropriate to apply on directly.

To conquer the challenge of identifying the sarcophagid specimens, mitochondrial DNA-based method had been developed and shown its validity. For example, Wells [6] described the usefulness of cytochrome oxidase subunit one (COI) for species identification of the Sarcophagidae from Canada and USA. Zehner [10] analyzed the specific DNA region at COI and ND5 of European sarcophagid flies. By using the COI DNA fragment, Guo identified forensically important sarcophagid flies in China [11]. Meiklejohn [9] sequenced the COI barcode fragment of Australian sarcophagid samples, and they found that the DNA sequence variation among Parasarcophaga dux (Thomson, 1869) is high (4.6%). For the small island of Taiwan, without large geographical separation, it is meaningful to know the COI DNA sequence variation among the samples of P. dux. It is also interesting to know the relationship between DNA sequence diversity and morphological feature of P. dux. In this paper, the correlation between genotype (COI DNA variations) and phenotype (morphological characteristics) was also discussed.

MATERIALS AND METHODS

Samples
The fly specimens were collected from pig carrions in northern and southern Taiwan during different seasons. Eleven imitate experiments were performed by disposing pig corpses on different areas, four in Taipei county, six in Kaohsiung county and one in Cha-I county. The adult flies were collected by insect-sweeping net and then were preserved in 75 % alcohol. Sarcophagid flies were identified according to the key described by Smith [12]. Species typing of sarcophagid flies were carried out based on the identification keys reported by Rokuro [13] and Fan [14, 15].

DNA extraction
Total genomic DNA was extracted from one leg of fly samples. Each leg was ground into powder using disposable plastic pestles inside 1.5 ml microcentrifuge tubes immersed in liquid nitrogen. DNA extraction was performed by using QIAamp DNA kit (QIAGEN Inc, Valencia, CA, USA).

Amplification and sequencing
The DNA so obtained was used as the template for PCR reactions. A region of mitochondrial cytochrome oxidase b subunit I (COI) gene was amplified using primers C1-J-1718 (5’-GGAGGATTGGAAATT- GATTAGTTCC-3’) and TL2-N-3014 (5’-TCCAATGCACATATGCCATATTA-3’) [16], and a fragment from subunit II gene (COII) by using primers C2-J-3138 (5’-AGAGGCTCTTCTTATAGAA-3’) and TK-N-3775 (5’GAGACCATTACTTGCTTTCAAGTCATC-3’) [16].

The PCR reaction mixture consisted of 5 μl 10x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl2), 8 μl of 1.25 mM dNTP mix, 5 μl 1.5 μM forward and reverse primers, 2.5 units of Taq DNA polymerase, 10 ng of DNA and distilled water to a final volume of 50μl. The PCR conditions were as follows: 35 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 1 minute.

Prior to the sequencing reaction, the PCR products were purified using a QIAquick® PCR Purification Kit (QIAGEN Inc, Valencia, CA, USA). Cycle sequencing was performed using 3.0 μl of ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction solution with AmpliTaq FS DNA polymerase (PE Applied Biosystems, Foster City CA, USA), 10 pmol of forward or reverse primer, 2.3 μl of purified PCR product and sterile distilled water to final volume of 10 μl for each sample. For cycle sequencing, a Perkin Elmer 9600 thermal cycler was used under the following conditions: 25 cycles at 96°C for 30 s, 50°C for 15 s, 60°C for 4 minutes. After sequencing, each sample was added to a sephadex-G-50 column (spin 50-mini-column, BioMax Inc. Odenton, MD, USA) and centrifuged at 1500 × g for 3 min. Each purified sample was recovered at the bottom of the collection tube and dried in a vacuum centrifuge. Automated DNA sequencing was performed on an ABI 3100 sequencer.

DNA sequencing for the mitochondrial COII DNA fragment between nucleotide position (np) 1718 and np 3014 was performed for all the sarcophagid samples. The COII DNA fragment between np 3138 and np 3775 was used for species confirmation of the Sarcophagidae.
**Neighbor-Joining tree analysis**

The pairwise distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The neighbor-joining analysis was performed using Tamura-Nei mode of substitution and 500 bootstrap conducted using MEGA 4.0 [17, 18]. DNA sequence of Chrysomya megacephala (Fabricius, 1784) was assigned for out group in all analysis.

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**RESULTS AND DISCUSSION**

Among the fly specimens from mock experiments, 843 were identified to be blow flies, 47 to be sarcophagid flies, and about 200 flies to be Mucidae or others. For the 47 sarcophagid flies, seven species, *L. crassipalpis*, *P. misera*, *P. dux*, *L. ruficornis*, *B. pergrina* (Robineau-Desvoidy, 1830), *B. formosensis* Kirner & Lopes, 1961, and *Seniorwhitea princeps* (Wiedemann, 1830) were recognized by microscopically observing the morphology at head, thorax, and abdomen. Out of 47 sarcophagid flies, 19 were identified to be *Parasarcophaga dux*.

For the 47 sarcophagid samples, agreement with the species classification results from morphological observation, the DNA analysis results of the COI DNA sequence diversity of seven inter-species are shown in Table 1. The nucleotide diversity was over 5% between the following four species: *L. crassipalpis*, *L. ruficornis*, *P. dux* and *P. misera*. The lowest divergence (2.2%) appeared between *B. pergrina* and *B. formosensis*. The COI DNA sequence diversity within the intra-species were less than 0.8% for *L. crassipalpis*, *P. misera*, *L. ruficornis*, *S. princeps*, 1.2% for *B. pergrina*, and 1.8% for *P. dux* (see Table 2).

The COI DNA sequence diversity between the seven species of forensically important sarcophagid flies in Taiwan ranged from 2.2% to 11.4%. The inter-species diversity in this study was higher than that of sarcophagid flies reported from China [11], where the level of inter-specific variation varied from 5% to 9%. The different inter-specific variation between the two might be due to different species analyzed. For example, the two species (*B. pergrina* and *B. formosensis*) with high similarity in Taiwan were not included in that paper [11]. Careful examination for the samples of *B. pergrina* and *B. formosensis* revealed that they still have minor morphological difference on the genae. Specifically, *B. pergrina* had black genae with golden to silvery pollinosity and with several numbers of yellowish hairs posteriorly, and contrarily, all hairs were black for *B. formosensis* [14]. The high similarity of the two species indicated that they might be close species with nearest origin.

The threshold value of mitochondrial DNA divergence for species identification had been discussed by several entomologists. Wells [19, 20] suggested that the level of nucleotide divergence of COI DNA sequence between different species of blow flies or between different species of flesh flies was higher than 3%, within same species less than 0.8%. However, Nelson [21]
reported that the intra-species percentage divergences of COI DNA barcodes ranged between 0–0.612%, in comparison with interspecies variation being 0.458% to 9.254% for Chrysomya species from the east coast of Australia. The data showed that some intra-species had high DNA variation, exceeding over that of inter-species. The low interspecies sequence divergence was found among the closely related species, which were believed to be recent origin. Similarly, Harvey [22] analyzed calliphorid species based on a DNA fragment of 1167 bp to geographical separation. Therefore, it is difficult to get an unambiguous threshold value of DNA divergence for species identification.

It is noteworthy that the highest DNA variation (1.8%) within the P. dux intra-species was obtained merely in Taiwan. No significant intra-specific variation was observed within S. dux samples in China [11]. This may be due to that the length of COI DNA fragment studied in their study was only 272bp. Our result showed that the DNA variation was lower than that of P. dux in Australia (4.6%) [9]. Such a difference could be from a geographical separation factor, Taiwan (small island) versus Australia (large area). Based on these data, we speculated that the DNA variation in intra-species of P. dux might be high for many places in the world.

Detailed DNA sequence variation of the 19 specimens of P. dux is shown in Table 3. Based on sequence similarity, they can be classified into three subgroups: A, B, and C. Obviously, higher DNA variance was observed between the samples of different subgroup compared with those within same subgroup. Higher similarity sequence was observed among the samples within the group A, compared with that of group B and C. According to the literature [14], either black or orange yellow 2nd genital segment was found in P. dux flies. In subgroup A, all the four samples were found to be black at 2nd genital segment (see Fig. 1). There were two haplotypes detected with a variable site at np 2934. Within the subgroup B, three haplotypes with eleven variable sites were found. The eight samples (B1, B2, B4, B6, B7, B8, B9, and B10) were of the same haplotype and had black genital segment. Based on the DNA sequence, the other two samples (B3 and B5) were classified as two separate haplotypes and were found to be orange yellow at genital segment. Four haplotypes with only two variable sites were detected within the subgroup C (most to be orange yellow genital segment), in which the sample C4 with black genital segment had a specific variable site at np 2934.

 Neighbor-joining tree relationships among the 19 COI sequences of P. dux are shown in figure 2, indicating that the 19 DNA sequences can also be divided into three subgroups (A, B, C), and that B3, B5 were close to subgroup C (with orange yellow color) and C4 was close to subgroup B (with black color). Based on these results, we concluded that there could be a relationship between the color variation at 2nd genital segment and COI DNA sequence diversity among the P. dux.

Table 2 showed that nucleotide divergence was
1.8% (between subgroup A and B), 1.6% (A and C), and 1.2% (B and C), and that the divergence within subgroup A, B, and C was less than 0.1%, 0.7%, and 0.4%, respectively. The extent of nucleotide divergence (less than 1.8%) of P. dux indicated that the three subgroups still can be the same species, but be regarded as different subgroups within same species. For further distinguishing the close groups in P. dux species, we also sequenced COII (np3138-3775, data not shown), revealing that the nucleotide divergence was 1.3% (between group A and B), 1.8% (A and C), and 0.5% (B and C). These results supported the high sequence diversity among the three subgroup flies within P. dux species. Using the neighbor-joining tree method, Meiklejohn [9] found two separate clusters in the Australian P. dux samples, however, lacking of further confirmation.

CONCLUSION

This study offered the DNA analysis data and morphological characteristics of sarcophagid flies collected in Taiwan. Three significant results were obtained. First, seven species of forensically important sarcophagid flies were established in Taiwan. Second, the COI DNA sequence diversity (1.8%) in intra-species of P. dux was higher than those in other sarcophagid species, but was lower than that in Australia (4.6%). Third, we initially found that there might be a relationship between the COI DNA sequence variations and morphological features at the shape of pregonite as well as the color of 2nd genital tergite.

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