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The utility of Mitochondrial DNA fragments for genetic identification of forensically important sarcophagid flies (Diptera: Sarcophagidae) in China

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Abstract. Species-diagnostic anatomical characters of fleshflies are not known for most immature stages or even adults, and an existing key may be incomplete or difûcult for nonspecialists to use. The use of sarcophagids for PMI estimations has been greatly hampered by their highly similar morphological characters. DNA-based method can be used as a supplemental means of morphological method in identification of forensically important sarcophagid flies. However, relying solely on single DNA fragment for delimiting species is considered to be unreliable, especially when the fragment was small. Sequence data of selected regions of the cytochrome oxidase subunit two (COII) and 16S ribosomal RNA (16S rRNA) genes of the most important Chinese fleshfly taxa associated with cadavers are presented, which can be instrumental for implementation of the Chinese Sarcophagidae database. Phylogenetic analysis of the sequenced segments showed that all sarcophagid specimens were properly assigned into five species, which indicated the possibility of separation congeneric species with the short fragments.

INTRODUCTION

Insects attracted to cadavers may provide important indications of the postmortem interval (PMI). In addition to blow flies (Diptera:Calliphoridae), which are the predominant insect group found in human corpses and thus play an important role in forensic investigations; fleshflies (Diptera: Sarcophagidae) have also been commonly found in these cases (Byrd & Castner, 2009; Sukontason *et al.*, 2010). However, the use of sarcophagids as PMI estimators has been greatly hampered by their highly similar morphology and inadequate documentation of their thermobiological histories (Wells *et al.*, 2001; Zehner *et al.*, 2004). To implement the use of sarcophagids for PMI estimation, a method for easy yet accurate species-level identification at any life stage is required, followed by thermobiological studies (Meiklejohn *et al.*, 2010).

DNA-based method can be used as a supplemental means of morphological method in identification of forensically important flies (Wells & Stevens, 2008). In the last decade, various segments of the cytochrome oxidase subunit one (COI) region have been used to discriminate forensically important sarcophagid species (Wells *et al.*, 2001; Zehner *et al.*, 2004; Saigusa *et al.*, 2005; Song *et al.*, 2008; Meiklejohn *et al.*, 2010; Tan *et al.*, 2010; Guo *et al.*, 2010c; Mazzanti *et al.*, 2010). Even partial sequences of this COI gene have been proven to have sufcient discrimination power (Wells *et al.*, 2001;

Zehner *et al.*, 2004; Saigusa *et al.*, 2005; Song *et al.*, 2008; Guo *et al.*, 2010c; Mazzanti *et al.*, 2010). However, with the development of molecular identification, various discussions about the feature of the mitochondrial DNA (mtDNA) fragments were raised. The dangers of relying on a single locus are illustrated by several studies (Stevens *et al.*, 2002; Wells *et al.*, 2007). If only a small fragment of DNA is sampled, it may fail to produce an accurate representation of the total genetic variability in that gene. This could result in misrepresentation of the intra- and interspecic divergences between closely related species, leading to an inaccurate species delimitation (Roe & Sperling, 2007). Suggested targets other than COI included the gene for cytochrome oxidase subunit two (COII) (Stevens & Wall, 2001; Guo *et al.*, 2010b), the ribosomal internal transcribed spacer regions (Nelson *et al.*, 2008), the NADH dehydrogenase subunit 5 (Zehner *et al.*, 2004) and the gene for 16S ribosomal RNA (Wang *et al.*, 2010). Among these genes, COII had been sequenced over the widest variety of taxa with homologous sequences available for nearly all orders (Caterino *et al.*, 2000), and it was particularly useful in evolution studies, population genetics and systematics due to the relatively high degree of variation in the region molecular systematic study (Ying *et al.*, 2007). The 16S rRNA accumulates mutations more rapidly than the nuclear DNA genes and can infer relationships beneath the family level within insects (Simon *et al.*, 1994).

Although the sample sizes were small, the short COII and 16S rRNA fragments were successfully used to identify Sarcophagidae in this study. The composition of each species has its own local characteristics. To the best of our knowledge, the data of the COII and 16S rRNA gene of fleshflies form China are inadequate. This study evaluates the suitability of the 637 bp COII and 555 bp 16S rRNA fragments for identification of fleshfly species, under experimental conditions prior to application in Chinese criminal investigations. The two loci of 30 flesh fly specimens were sequenced and deposited in GenBank to expand local databases.

MATERIALS AND METHODS

Specimens

Thirty adult fleshfly specimens were obtained during the months of June to September in China from 2009 to 2010. Another two dried adult specimens of *Musca domestica* (Diptera: Muscidae) were obtained from Xi'an (Shannxi) and Yongzhou (Hunan) in the year 2009. Collection data for all specimens used in this study were listed in Table 1. All samples were collected using traps baited with animal remains (rabbit, dog, or pig). Samples were subsequently air-dried at room temperature or stored in 70% ethanol at -20ºC. All adult flies were identified using morphological keys (Xu & Zhao, 1996; Lu & Wu, 2003) by entomologists in Hunan Agricultural University.

DNA extraction

The mtDNA of all samples were extracted using the CTAB method (Guo *et al.*, 2010a). To avoid possible contamination of fly DNA with DNA from ingested protein and gut parasites, only the thoracic muscle of each insect was used as a source of DNA, and the head and abdomen of each specimen was retained to check the identity and for a further molecular analysis.

PCR

The PCR reaction volume was 25ml, containing 1-5ml (20-40ng) of template DNA, 12.5ml 2×GoTaq® Green Master Mix (Promerga, Madison, WI, USA) (4ml dNTP (1mmol/ml), 1.0u Taq polymerase, 2.5ml 10×buffer (Mg2+1.5mmol/l)), 0.25-2.5ml each primer (10mM), Nuclease-Free Water added to a total volume of 25 ml.

The following primers were used:

- COII (C2-J-3138): 5'-AGAGCCTCT CCTTTAATAGAACA-3'
- COII (TK-N-3775): 5'-GAGACCATT ACTTGCTTTCAGTCATC-3'

according to Wells & Sperling (2001) and:

- 16S rRNA (LR-J-12887): 5'-CCGGTC TGAACTCAGATCACGT-3'
- 16S rRNA (LR-N-13398): 5'-CGCCTG TTTAACAAAAACAT-3'

according to Simon *et al* (1994).

Table 1. Locality and reference data of specimens newly sequenced for this study

No.	Species	Accession number 16S	COa!	Locality and coordinates	Date
1	*P. dux* (Thompson, 1869)	JF416584	JF416614	Kunming, Yunnan [102:50E, 24:48N]	2010
2		JF416585	JF416615	Xining, Qinghai [101:49E, 36:37N]	2010
3		JF416586	JF416616	Urumqi, Sinkiang [87:36E, 43:46N]	2010
4		JF416587	JF416617	Chifeng, Inner Mongolia [118:38E, 43:24N]	2010
5		JF416588	JF416618	Beian, Heilongjiang[126:31E, 48:15 N]	2010
6		JF416589	JF416619	Nanping, Fujian [118:10E, 26:38N]	2010
7		JF416590	JF416620	Xining, Qinghai [101:49E, 36:37N]	2010
8	*P. albiceps* (Meigen, 1826)	JF416591	JF416621	Qiqihaer, Heilongjiang [123:97E, 47:33N]	2010
9		JF416592	JF416622	Xining, Qinghai [101:49E, 36:37N]	2010
10		JF416593	JF416623	Kunming, Yunnan [102:50E, 24:48N]	2010
11		JF416594	JF416624	Changsha, Hunan [112:59E 28:12N]	2010
12		JF416595	JF416625	Chifeng, Inner Mongolia [118:38E, 43:24N]	2010
13	*B. peregrina* (R-D, 1830)	JF416596	JF416626	Kunming, Yunnan [102:50E, 24:48N]	2010
14		JF416597	JF416627	Changsha, Hunan [112:59E 28:12N]	2010
15		JF416598	JF416628	Xining, Qinghai [101:49E, 36:37N]	2010
16		JF416599	JF416629	Qiqihaer, Heilongjiang [123:97E, 47:33N]	2010
17		JF416600	JF416630	Qiqihaer, Heilongjiang [123:97E, 47:33N]	2010
18		JF416601	JF416631	Urumqi, Sinkiang [87:36E, 43:46N]	2010
19		JF416602	JF416632	Chifeng, Inner Mongolia [118:38E, 43:24N]	2010
20		JF416603	JF416633	Beian, Heilongjiang[126:31E, 48:15 N]	2010
21		JF416604	JF416634	Nanping, Fujian [118:10E, 26:38N]	2010
22	*H. melanura* (Meigen, 1826)	JF416605	JF416635	Xining, Qinghai [101:49E, 36:37N]	2010
23		JF416606	JF416636	Qiqihaer, Heilongjiang [123:97E, 47:33N]	2010
24		JF416607	JF416637	Qiqihaer, Heilongjiang [123:97E, 47:33N]	2010
25		JF416608	JF416638	Suihua, Heilongjiang [126:98E, 46:67N]	2010
26		JF416609	JF416639	Suihua, Heilongjiang [126:98E, 46:67N]	2010
27	*P. gravelyi* (Senior-White, 1924)	JF416610	JF416640	Zhangjiajie, Hunan [114:51E, 38:04N]	2010
28		JF416611	JF416641	Zhangjiajie, Hunan [114:51E, 38:04N]	2010
29		JF416612	JF416642	Zhangjiajie, Hunan [114:51E, 38:04N]	2010
30		JF416613	JF416643	Zhangjiajie, Hunan [114:51E, 38:04N]	2010
31	*M. domestica* (Linnaeus, 1758)	GQ396684	GQ356681	Baotou, Inner Mongolia [118:38E, 43:24N]	2009
32		GQ396693	GQ356683	Yongzhou, Hunan [111:61E, 26:42N]	2009

A Perkin-Elmer 9600 thermocycler was used. For COII and 16S rRNA the following conditions for touch-down PCR were used: initial step at 94ºC (4 min), continued for 32 cycles of 94ºC (30s) and 55ºC (45s for mtDNA annealing) and 72ºC (30s). An elongation of PCR products by 72ºC for 5min completed the reaction.

Sequencing

Vertical non-denaturing polyacrylamide gel electrophoresis was used to isolate PCR products, which were then purified using a QiaQuick PCR Purification Kit (Qiagen). Columns cycle sequencing was performed on both forward and reverse strands using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit by ABI PRISM 3730 (Applied Biosystems, Foster City, USA) with BigDye terminator v3.1 as the sequencing agent. Sequence chromatograms were edited, and discrepancies between forward and reverse sequences were resolved using Sequence Navigator (v1.01, Applied Biosystems, Foster City, USA).

Sequence analysis and phylogenetic tree construction

Since the sequences did not contain any insertions or deletions, all resultant sequences in this study were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The obtained sequences have been deposited in GenBank by Sequin (http://www.ncbi.nlm.nih.gov/Sequin/index.html) and their accession numbers are listed in Table 1. To identify species, the COI and 16S

rRNA sequences were compared with Dipteral sequences on the NCBI web site via the BLASTn function.

The evolutionary history was inferred using the UPGMA method (Sneath & Sokal, 1973). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2007) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 637 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2004).

RESULTS AND DISCUSSION

All 30 samples were identified by entomologists into five distinct morphological types belonging to 4 genera (*Boettcherisca peregrina*, *Helicophagella melanura*, *Parasarcophaga albiceps*, *Parasarcophaga dux* and *Phallosphaera gravelyi*) using traditional morphological characteristics. Sampling multiple specimens from across the most known geographic range of the species was included. The *B. peregrina*, *H. melanura*, *P. albiceps*, and *P. dux* are widespread fleshfly taxa associated with cadavers in China. Moreover, *S. dux* and *S. peregrina* are the species of greatest forensic importance in China (Hu & Wang, 2000; Guo *et al.*, 2010b; Cai, 2011) and nearby countries (Sukontason *et al.*, 2010). Four specimens of *P. gravelyi* were collected near to the animal carcasses, when we conducted succession experiments in mid-slope of Tianmen Mountain (located in Zhangjiajie and 1200 meters height above sea level). The *P. gravelyi* specimens were included for purposes of phylogenetic comparisons.

All flies were correctly assigned into five species with monophyletic separation in the two trees (Fig. 1 and Fig. 2). As outgroup, the two specimens of *M. domestica* clustered together and were clearly separated from the sarcophagid mitotypes (Fig. 1 and Fig. 2). Phylogenetic analysis performed based on the COII sequence shows that different branches were formed sharing low supporting values for the *B. peregrina*, *H. melanura*, *P. albiceps*, and *P. dux* (Fig. 1). In the genus of *Parasarcophaga*, the two species *P. dux* and *P. albiceps* clustered together with a supporting bootstrap of 62%, indicating the efficacy of COII to identify the species from same genus of Sarcophagidae family. In the Figure 2, the monophyletic separation of the five sarcophagid species in the phylogenetic tree examined by 16S rRNA confirmed the sufficient resolution of the genetic marker. The five species groups of Sarcophagidae were separated and the bootstrap values were all high. However, in the genus level, the *P. albiceps*, *P. dux* and *P. gravelyi* cluster together directly, while *B. peregrina* and *H. melanura* cluster together with a supporting bootstrap of 96% indicating the ability of this partial 16S rDNA region to identify the species from same genus was not as efficient as that of the partial COII region. This may be related to the selected region of the 16S rRNA gene, and more sarcophagid species from more locations should be studied in the future.

In Table 2 (below diagonal), all values for maximum intra specific variations of the sarcophagid species were no more than 3%. However, the small sample size makes it difficult to analyze the high intra specific variations. More samples from different locations of China need to be studied in the future. The inter specific variations between sarcophagid species varied from 7% to 11%. The inter specific variations between species of different genera were higher than that from the same genus, which indicated the efficacy of COII to identify the species from different

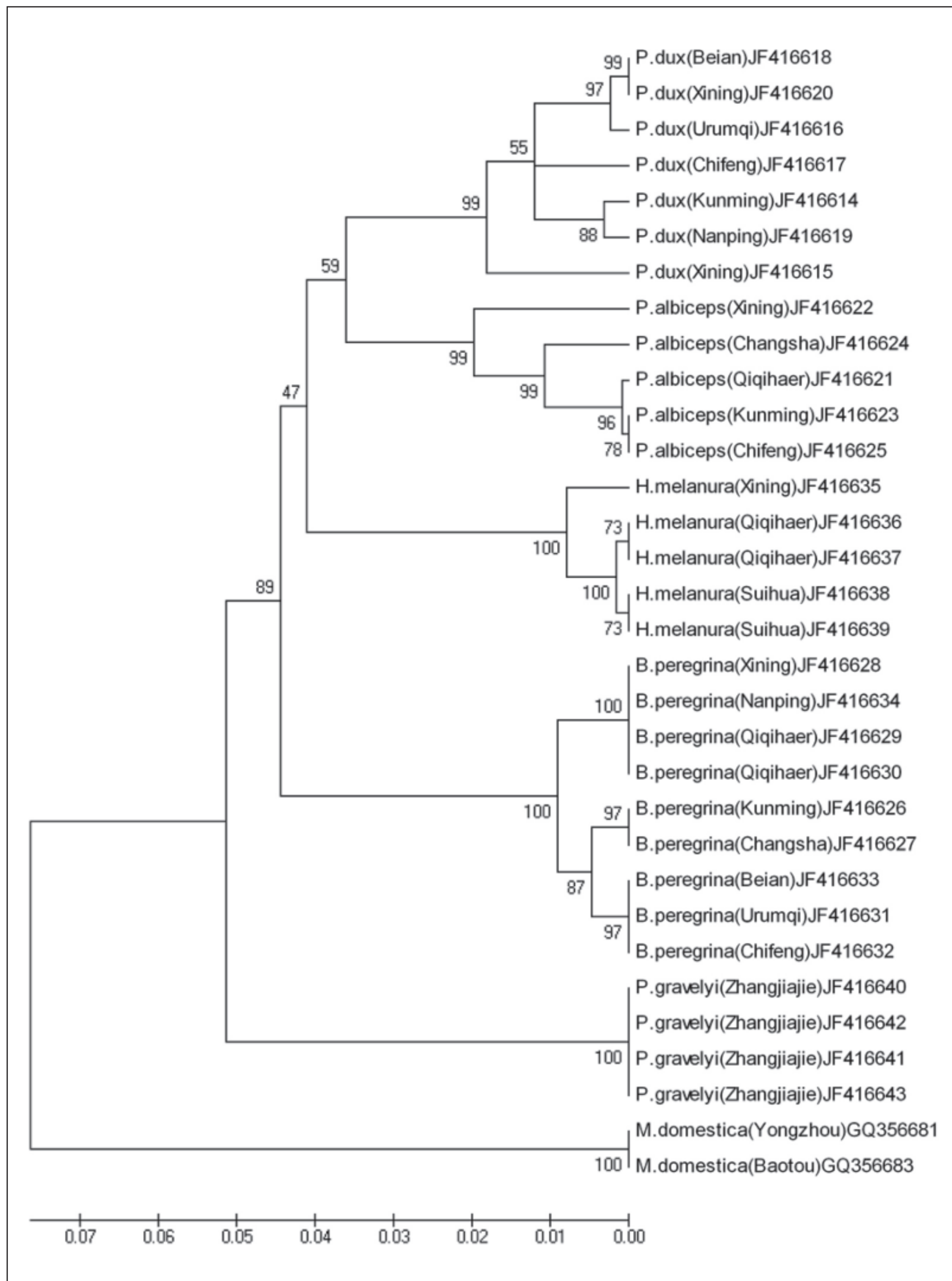

Figure 1. Evolutionary relationships of 30 taxa using the UPGMA method based on a 637 bp region of the CO II gene. Numbers on branches indicate the support value. Morphological species identification, voucher ID and City are given in specimen label. Two *Musca domestica* samples from family Muscidae are included as outgroup

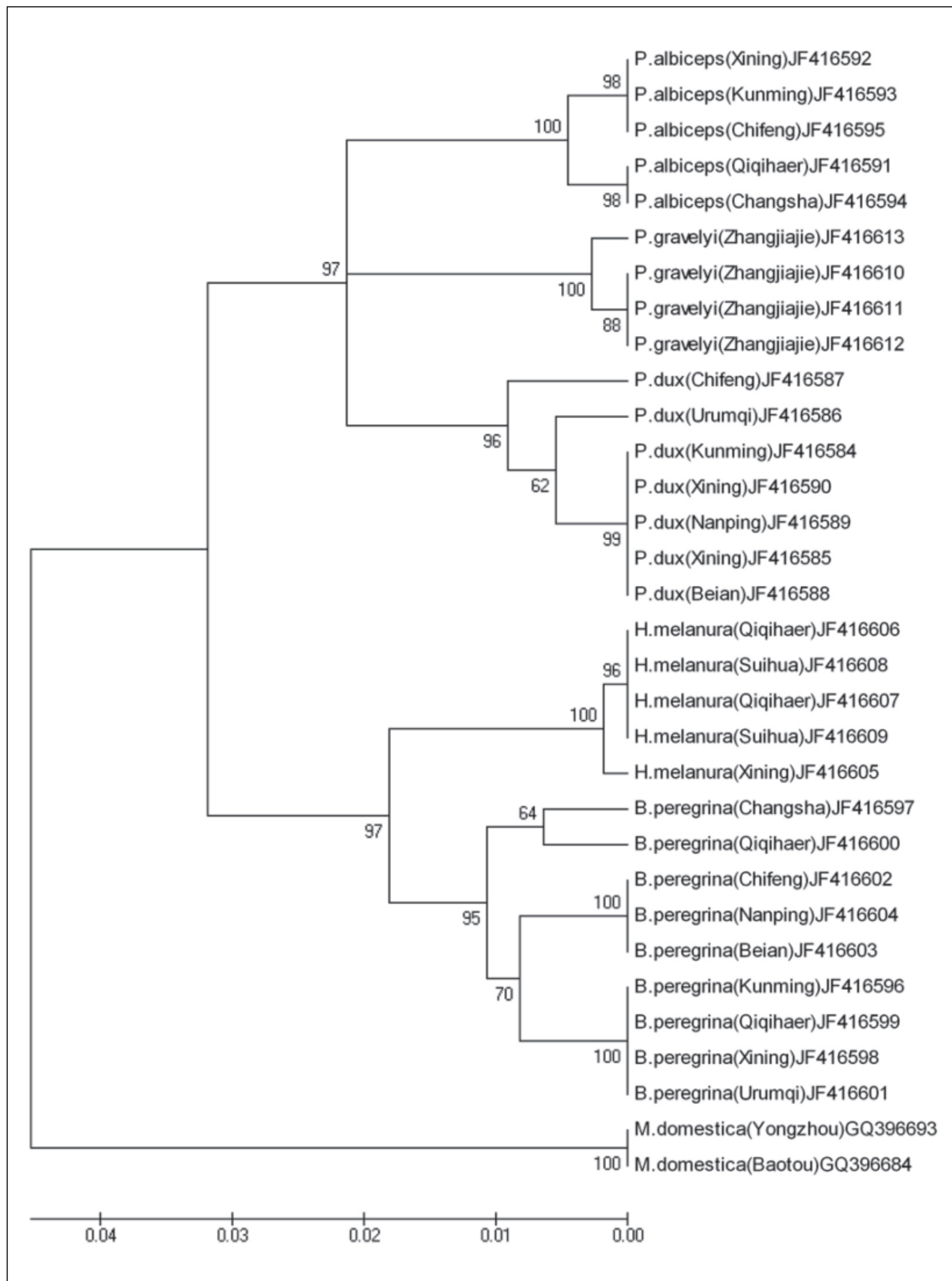

Figure 2. Evolutionary relationships of 30 taxa using the UPGMA method, based on a 555 bp region of the 16S rRNA gene. Numbers on branches indicate the support value. Morphological species identification, voucher ID and City are given in specimen label. Two *Musca domestica* samples from family Muscidae are included as outgroup

Table 2. Calculated intraspecific and interspecific variations expressed as a percentage of the 555bp CO II (below diagonal) and 637 bp 16S rRNA (above diagonal) data

No.	Species	No. S[a]	Range and mean[b]		1	2	3	4	5
			COII	16S rRNA					
1	*B. peregrina*	9	0-1 (1)	0-3 (1)	–	7	4	7	7
2	*P. albiceps*	5	0-3 (2)	0-1 (0)	8	–	5	4	3
3	*H. melanura*	5	0-2 (1)	0-1 (0)	8	9	–	7	6
4	*P. dux*	7	0-3 (2)	0-3 (1)	8	6	7	–	5
5	*P. gravelyi*	4	0-0 (0)	0-0 (0)	11	11	9	9	–

[a] No. S = Number of specimens with Tamura

[b] Range and mean = Range from the minimum intraspecific variation to the maximum intraspecific variation. And the within group means were listed in brackets

genera of Sarcophagidae. Furthermore, the interspecific variation between *P. dux* and *P. albiceps* was larger than 5%, which could distinguish these species of the same genus *Parasarcophaga*. Table 2 (above diagonal) showed that the small amplicon size and highly conservative region made this selected 16S rRNA region unable to supply sufficient amount of information within species. However, the mean levels of divergence between these five sarcophagid species ranged from 3% to 7%. The interspecific variation between species of some species were less than 5%, indicating that relying solely on this short 16S rRNA fragment for delimiting species is dangerous, especially for the species of same genus. Although the 16S rRNA accumulates mutations more rapidly than the nuclear DNA genes, compared to COII sequence, this selected 16S rRNA region is relatively high conserved. Therefore, the combination of different fragments would be more scientific and reliable.

We assessed the COII and 16S rRNA sequences as potential markers for the identification of Sarcophagidae family flies from China. The phylogenetic analysis results of these specimens from different regions provided us the possibility of applying these two genetic markers as an identication tool for the common Sarcophagidae species from China. The results indicated that the used technique is as effective as morphological method in identification of Sarcophagidae family species, while, in order to acquire correct identification, the morphologic method needs expertise in specialized taxonomy (Saigusa *et al.*, 2005). Therefore, the used technique can be used as supplementary means of morphological method in identification of sarcophagids, as the technology used mtDNA is easier to perform and saves more time for forensic scientists within their routine work.

Forensically important species can be separated using short (200-300 bp) mtDNA fragments such as can be obtained from even very degraded DNA (Wang *et al.*, 2010; Guo *et al.*, 2010a, 2010b, 2010c; Cai, 2011). However, there is no single agreed-upon locus for DNA-based identication of forensic insects, and it is not clear that there could be (Wells & Stevens, 2008). Although longer fragments may minimize stochastic variation across taxa and be more likely to reect broader patterns of nucleotide divergence (Roe & Sperling, 2007), shorter fragments have many advantages, such as quick, easy and economical. Up to now, many projects, such as Barcodes of Life (http://barcoding.si.edu/DNABarCoding.htm), are still opting to use only relatively short DNA fragments. Relying upon a single short DNA fragment for identifying is dangerous, as the results may be misleading (Wells *et al.*, 2007). Thus, the combination of short mtDNA fragments was used in this study, and various

regions of DNA should be studied to find the best region with truly diagnostic nucleotide changes in the future study.

The DNA-based method will be increasingly used in forensic entomology research and case work throughout the world. Designation of a piece of mtDNA fragment to identify and delineate the insect character is one of the key features of this method. In this study, the processes of obtaining the 555-bp 16S rRNA and 637-bp COII fragments were simple and the error of sequencing was reduced. Moreover, the identification results of this study were comparable to the ones using the longer fragments, which showed potentiality of the short fragments as the discriminatory tool in identification of Sarcophagidae. In the future study, numerous sequences should be obtained to maximize sampling of diversity within and between species, so that overlap of intra- and interspecic divergence can be more accurately identied.

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REFERENCES

Byrd, J.H. & Castner, J.L. (2009). Insects of forensic importance. *Forensic Entomology – The Utility of Arthropods in Legal Investigations* (ed. By J.H. Byrd & J.L. Castner), pp. 39–127. CRC Press, Boca Raton.

Cai, J.F. (2011). *Modern and practical forensic entomology.* People's Medical Publishing House, Beijing.

Caterino, M.S., Cho, S. & Sperling, F.A.H. (2000). The current state of insect molecular systematics: a thriving Tower of Babel. *Annual Review of Entomology* **45**: 1-54.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-79.

Guo, Y.D., Cai, J.F., Su, R.N., Chang, Y.F., Lan, L.M. & Wen, J.F. (2010a). Comparison of mtDNA extraction from different parts of sarcosaphagous insects. *Fa Yi Xue Za Zhi* **26**: 336-339.

Guo, Y.D., Cai, J.F., Li, X., Xiong, F., Su, R.N., Chen, F.L., Liu, Q.L., Wang, X.H., Chang, Y.F., Zhong, M., Wang, X. & Wen, J.F. (2010b). Identification of the forensically important sarcophagid flies *Boerttcherisca peregrina*, *Parasarcophaga albiceps* and *Parasarcophaga dux* (Diptera: Sarcophagidae) based on COII gene in China. *Tropical Biomedicine* **27**: 451-460.

Guo, Y.D., Cai, J.F., Wang, X.H., Lan, L.M., Liu, Q.L., Li, X., Chang, Y.F., Ming, Z., Wang, X. & Wen, J.F. (2010c). Identification of forensically important sarcophagid flies (Diptera: Sarcophagidae) based on COI gene in China. *Romanian Journal of Legal Medicine* **18**: 217-224.

Hu, C. & Wang, J.F. (2000). Diptera with forensic importance. In: Hu C, Min JX (ed) *Forensic Entomology*. Chongqing publishing house, Chongqing, pp 96-160.

Lu, B.L. & Wu, H.Y. (2003). Classification and identification of important medical insects of China. Henan Science and Technology Publishing House, Zhengzhou.

Mazzanti, M., Alessandrini, F., Tagliabracci, A., Wells, J.D. & Campobasso, C.P. (2010). DNA degradation and genetic analysis of empty puparia: Genetic identication limits in forensic entomology. *Forensic Science International* **195**: 99-102.

Meiklejohn, K.A., Wallman, J.F. & Dowton, M. (2010). DNA-based identification of forensically important Australian Sarcophagidae (Diptera). *International Journal of Legal Medicine*DOI 10.1007/s00414-009-0395-y).

Nelson, L.A., Wallman, J.F. & Dowton, M. (2008). Identication of forensically important *Chrysomya* (Diptera: Calliphoridae) species using the second ribosomal internal transcribed spacer (ITS2). *Forensic Science International* **177**: 238–247.

Roe, A.D. & Sperling, F.A.H. (2007). Patterns of evolution of mitochondrial cytochrome c oxidase I and II DNA and implications for DNA barcoding. *Molecular Phylogenetics Evolution* **44**: 325-345.

Saigusa, K., Takamiya, M. & Aoki, Y. (2005). Species identification of the forensically important flies in Iwate prefecture, Japan based on mitochondrial cytochrome oxidase gene subunit I (COI) sequences. *Legal Medicine (Tokyo, Japan)* **7**: 175-178.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**: 651-701.

Sneath, P.H.A. & Sokal, R.R. (1973). Numerical Taxonomy. Freeman, San Francisco.

Song, Z.K., Wang, X.Z. & Liang, G.Q. (2008). Phylogenetic relationships among 15 sarcophagid fly species (Diptera: Sarcophagidae) based on partial sequences of mitochondrial cytochrome b and cytochrome oxidase subunit 1 genes. *Acta Entomologica Sinica* **51**: 298-306.

Stevens, J. & Wall, R. (2001). Genetic relationships between blowies (Calliphoridae) of forensic importance. *Forensic Science International* **120**: 116-123.

Stevens, J.R., Wall, R. & Wells, J.D. (2002). Paraphyly in Hawaiian hybrid blowy populations and the evolutionary history of anthropophilic species. *Insect Molecular Biology* **11**: 141-148.

Sukontason, K., Bunchu, N., Chaiwong, T., Moophayak, K. & Sukontason, K.L. (2010). Forensically important flesh fly species in Thailand: morphology and developmental rate. *Parasitology Research* **106**: 1055-1064.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596-1599.

Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)* **101**: 11030-11035.

Tan, S.H., Rizman-Idid, M., Mohd-Aris, E., Kurahashi, H. & Mohamed, Z. (2010). DNA-based characterisation and classication of forensically important esh ies (Diptera: Sarcophagidae) in Malaysia. *Forensic Science International* **199**: 43-49.

Wang, X.H., Cai, J.F., Guo, Y.D., Chang, Y.F., Wu, K.L., Liu, Q.L., Wang, J.F., Yang, L., Lan, L.M., Ming, Z., Wang, X., Song, C.Y., Liu, Y., Chen, Y.Q., Li, J.B., Dai, Z.H. & Peng, X. (2010). The availability of 16S rDNA (=RNA) gene for identifying forensically important blowflies in China. *Romanian Journal of Legal Medicine* **18**: 43-50.

Wells, J.D., Pape, T. & Sperling, F.A.H. (2001). DNA-based identification and molecular systematics of forensically important sarcophagidae (diptera). *Journal of Forensic Sciences* **46**: 1098-1102.

Wells, J.D. & Sperling, F.A.H. (2001). DNA-based identication of forensically important Chrysomyinae (Diptera: Calliphoridae), *Forensic Science International* **120**: 110-115.

Wells, J.D. & Stevens, J.R. (2008). Application of DNA-based methods in forensic entomology. *Annual Review of Entomology* **53**: 103-120.

Wells, J.D., Wall, R. & Stevens, J.R. (2007). Phylogenetic analysis of forensically important *Lucilia* ies based on cytochrome oxidase I sequence: a cautionary tale for forensic species determination. *International Journal of Legal Medicine* **121**: 229-233.

Xu, W.Q. & Zhao, J.M. (1996). Flies of China. Liaoning Science and technology press, Shenyang.

Ying, B.W., Liu, T.T., Fan, H., Wei, D., Wen, F.Q., Bai, P., Huang, J. & Hou, Y. P. (2007). The application of mitochondrial DNA cytochrome oxidase II gene for the identification of forensically important blowflies in Western China. *The America Journal of Forensic Medicine and Pathology* **28**: 308-313.

Zehner, R., Amendt, J., Schutt, S., Sauer, J., Krettek, R. & Povolny, D. (2004). Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). *International Journal of Legal Medicine* **118**: 245-247.