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PAPER

PATHOLOGY/BIOLOGY

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Identification of Forensically Important Sarcophagid Flies (Diptera: Sarcophagidae) in China, Based on COI and 16S rDNA Gene Sequences*

ABSTRACT: Insects attracted to cadavers may provide important indications of the postmortem interval (PMI). However, use of the flesh flies (Diptera: Sarcophagidae) for PMI estimation is limited as the species are often not morphologically distinct, especially as immatures. In this study, 23 forensically important flesh flies were collected from 13 locations in 10 Chinese provinces. Then, a 278-bp segment of the cytochrome oxidase subunits one (COI) gene and a 289-bp segment of the 16S rDNA gene of all specimens were successfully sequenced. Phylogenetic analysis of the sequenced segments showed that all sarcophagid specimens were properly assigned into four species (*Boerttcherisca peregrina* [Robineau-Desvoidy, 1830], *Helicophagella melanura* [Meigen, 1826], *Parasarcophaga albiceps* [Meigen, 1826], and *Parasarcophaga dux* [Thompson, 1869]) with relatively strong supporting values, thus indicating that the COI and 16S rDNA regions are suitable for identification of sarcophagid species. The difference between intraspecific threshold and interspecific divergence confirmed the potential of the two regions for sarcophagid species identification.

KEYWORDS: forensic science, forensic entomology, sarcophagid flies, cytochrome oxidase subunits one, 16S ribosomal DNA, species identification

The major mission of forensic entomology is to provide useful information for investigation of murders and suspicious deaths by helping to determine time, manner, and place of death (1). Necrophagous insects are attracted by decaying corpses and colonize them immediately after death (2). Insect larvae and adult insects found on human corpses provide important clues for the estimation of the postmortem interval (PMI), especially when information on the postmortem phenomena is not available (3). The Sarcophagidae or flesh flies (Diptera) comprise more than 2500 species in more than 100 genera globally, with many species being carrion breeders and initial corpse colonizers (4,5). Many sarcophagid species have the potential to be used to estimate the PMI (3,4,6). The larval stages of many species of Sarcophagidae are necrophagous, and for this reason, those species termed "flesh flies" are significant in forensic entomology, being second only to the Calliphoridae (Diptera) in terms of their usefulness (7). Death scenes from which flesh fly larvae have been recovered vary from ones with relatively fresh remains to much older mummified corpses (8). Moreover, the reproductive cycle of the sarcophagids makes them prospectively more reliable for PMI estimations compared with other initial dipteran colonizers (4,9). Flesh flies, such as genus Sarcophaga, can fly under inclement conditions that would prevent the Calliphoridae (10); thus, in the rainy seasons, flesh flies may be the first species to arrive on carrion.

Unambiguous and rapid sarcosaphagous insect species identification is an essential requirement for forensic investigations. Sarcophagine flies are notoriously difficult to identify because of their highly similar morphological appearance. For some sarcophagid species, it is only the adult males that can be identified with certainty (11). Furthermore, no suitable key for the identification of the immature stages of sarcophagid flies exists (4). Time-consuming rearing of the larvae to adults for identification may delay the criminal investigation or cause significant problems when rearing fails (3). Some authors suggested and demonstrated that mitochondrial DNA (mtDNA) sequences could be successfully employed to distinguish some species of the sarcophagine flesh flies (2,4,12–14). PCR amplification of selected regions of the mtDNA, sequence analysis of the amplicons obtained, and alignment of the data with reference sequences are the usual and recommended methods. Molecular methods can be carried out on any life cycle stage without further rearing and on dead, preserved, or live samples.

The DNA analysis of forensically relevant insects focuses mainly on the cytochrome oxidase subunits one (COI). Even partial sequences of this COI gene have been proven to have sufficient discrimination power (2,4,15–18), which makes it, in particular,

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suitable for forensic applications. However, some closely related species failed to be discriminated by COI sequence analysis (19–22). The main limitation to the use of COI sequence data has been the inability to distinguish between some closely related species (23). Under these circumstances, other sequences of forensically important flies from different geographical regions were examined, such as the nuclear encoded 28S rDNA sequence and the mitochondrial encoded 16S rRNA. The 28S rDNA sequences were used to identify some closely related species of Calliphoridae family and different results from the COI phylogenetic analyses were produced (24,25). Meanwhile, some studies indicate that 16S rDNA gene has potential as a discriminatory tool in identification of forensically important flies (26–28). In this study, a fragment (278 bp) of the COI mitochondrial gene and a fragment (289 bp) of the 16S rDNA gene from 23 Chinese Sarcophagid fly specimens belonging to the four forensically importance species were sequenced.

The knowledge of local fauna is very useful in forensic investigations, because data from other regions, which may have both different environmental and faunal characteristics, may not provide a sufficient degree of accuracy (20). The longitude and latitude span of China is so large that the climate condition and geographical environment differ considerably among provinces. However, molecular data available with respect to the sarcophagid flies are very limited in China. To the best of our knowledge, few studies on genetic identification of Sarcophagidae in China have been published. The goal of this study is to research the utility of COI and 16S rDNA for identification of four forensically important sarcophagid fly species in China, simultaneously to accumulate genetic data for the global study on Sarcophagidae.

Materials and Methods

Specimens

Six specimens of *Boerttcherisca peregrina*, four specimens of *Helicophagella melanura*, four specimens of *Parasarcophaga albiceps,* and nine specimens of *Parasarcophaga dux* were obtained from 13 different locations in China from 2007 to 2009. Another two dried adult specimens of *Musca domestica* (Diptera: Mascidae) were obtained from Xi'an (Shannxi) and Yongzhou (Hunan) in the year 2009. Collection information is listed in Table 1. Two kinds of baits were used: rat carcasses and pig carcasses. The animal carcasses were placed on the outdoor grassland. All the specimens were collected by hand net or tweezers, and subsequently, air-dried at room temperature, or stored in 70% ethanol at 20°C. All specimens were identified morphologically by expert entomologists through the use of relevant taxonomic keys (29,30).

The southernmost collection location is Wanning located in Hainan Province, SA (18°80′, 110°39′), and the northernmost and westernmost location is Urumqi in Sinkiang Municipality, SA (43°46′, 87°36′). The easternmost collection location is Linyi located in Shandong Province, SA (35°05′, 118°35′). Although not every province is involved in this study, these samples almost represent the four main sarcophagid fly species throughout China.

DNA Extraction

mtDNA from all samples was extracted using the CTAB (cetyltriethylammnonium bromide) method (31). To avoid possible contamination of fly DNA with DNA from ingested proteins and eggs of gut parasites, the thoracic muscle of each insect was used as the source of DNA, whereas the head and abdomen were retained for further morphological and molecular analysis. DNA was resuspended in 50 μl of 1 × TE buffer (1 × TE buffer, pH 8.0; 10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at 4°C.

PCR Primers

The 278-bp fragment of the COI gene from all specimens was amplified using the primer pair C1-J-2495 (sense) (32) and C1-N-2800 (antisense) (33). All of the 16S rDNA sequences were aligned

TABLE 1—*Locality and reference data of specimens newly sequenced for this study.*

No.	Species	Accession Number		Locality and Coordinates	Date
		COI	16S		
1	*Sarcophaga peregrina* (Robineau-Desvoidy,1830)	HM037095	HM016588	Lanzhou, Gansu [103:83E, 36:06N]	2007
2		HM037097	HM016590	Jishou, Hunan [109:43E, 28:18N]	2008
3		HM037096	HM016589	Linyi, Shangdong [118:35E, 35:05N]	2007
4		GU145171	HM016587	Urumqi, Sinkiang [87:36E, 43:46N]	2009
5		GU145177	HM016592	Yongzhou, Hunan [111:61E, 26:42N]	2009
6		HM037098	HM016591	Changsha, Hunan [112:59E, 28:12N]	2009
7	*S. melanura* (Meigen, 1826)	HM037109	HM016607	Xining, Qinghai [101:49E, 36:37N]	2009
8		HM037110	HM016608	Yinchuan, Ningxia [106:91E, 38:46N]	2009
9		HM037111	HM016609	Shijiazhuang, Hebei [114:26E, 38:03N]	2009
10		HM037112	HM016610	Shijiazhuang, Hebei [114:26E, 38:03N]	2009
11	*S. albiceps* (Meigen, 1826)	HM037099	HM016596	Shijiazhuang, Hebei [114:26E, 38:03N]	2009
12		GU145174	HM016594	Zhangjiajie, Hunan [114:51E, 38:04N]	2009
13		GU145197	HM016595	Hohhot, Inner Mongolia [111:38E, 40:48N]	2009
14		GU145178	HM016597	Zhangjiajie, Hunan [114:51E, 38:04N]	2009
15	*S. dux* (Thompson, 1869)	HM037107	HM016606	Datong, Shanxi [113:13E, 40:07N]	2007
16		HM037104	HM016602	Wanning, Hainan [110:39E, 18:80N]	2007
17		HM037103	HM016601	Changsha, Hunan [112:59E, 28:12N]	2007
18		HM037108	HM016605	Yongzhou, Hunan [111:61E, 26:42N]	2008
19		HM037106	HM016604	Xining, Qinghai [101:49E, 36:37N]	2009
20		HM037105	HM016603	Urumqi, Sinkiang [87:36E, 43:46N]	2009
21		HM037102	HM016600	Yongzhou, Hunan [111:61E, 26:42N]	2009
22		HM037100	HM016598	Xining, Qinghai [101:49E, 36:37N]	2009
23		HM037101	HM016599	Wanning, Hainan [110:39E, 18:80N]	2009
24	*Musca domestica* (Linnaeus, 1758)	GU145163	GU145238	Xi'an, Shannxi [108:91E, 34:23N]	2009
25		GU145164	GU145239	Yongzhou, Hunan [111:61E, 26:42N]	2009

using the sequence alignment program DNASTAR (MEGALIGN version 7.1.0; DNAStar Inc., Madison, WI). Conserved regions of the alignment were evaluated and marked. The most commonly occurring nucleotides at each position of the conserved sequence were selected and inputted in the primer design program PRIMER PREMIER 5.0 (PREMIER Biosoft International, Palo Alto, CA). The primer-binding site should lie entirely within the conserved region. And the general primer design rules were considered to avoid false priming and primer-dimer formation in cross-family PCR. A portion of 289-bp fragment of the mitochondrial 16sRNA gene was amplified and sequenced by using forward primer (5′-CGCTGTTATCCCTAAGGTAA-3′) and reverse primer (5′-CTGGTATGAAAGGTTTGACG-3′).

PCR Conditions

The PCR volume was 25 μL, containing 1–5 μL (20–40 ng) of template DNA, 12.5 μL 2 × GoTaq® Green Master Mix (Promega, Madison, WI)—4 μL dNTP (1 mmol/mL, 1.0 U Taq polymerase, 2.5 μL 10 × buffer [$Mg2^+$ 1.5 mM])—0.25–2.5 μL each primer (10 μM), nuclease-free water (Promega) added to a total volume of 25 μL. Amplifications were performed in a thermocycler (Perkin-Elmer 9600; Applied Biosystems, Foster City, CA) programmed with the following parameters: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, and a final elongation at 72°C for 5 min.

Sequencing

Vertical nondenaturing polyacrylamide gel electrophoresis was used to isolate PCR products, which were then purified using a Qiaquick PCR Purification kit (Qiagen, Hilden, Germany). 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, CA) with BIG DYE 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).

Because the sequences were protein coding and 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 sequences were compared with Dipteral sequences on the NCBI web site via the BLASTN function.

Phylogenetic Analysis

A total of 289 aligned sites for the 23 fragments of the mitochondrial 16S rDNA and a total of 278 aligned sites for the 23 fragments of the mitochondrial COI sequences were included in the analyses. Two *M. domestica* (Linnaeus, 1758) samples belonging to the family Mascidae were used as outgroup for phylogenetic analyses (Table 1).

To determine whether they were of mitochondrial origin or represented paralogous sequences in the nucleus, the sequences were tested using MEGA4 (34). Evolutionary history was inferred using the neighbor-joining method (35). The bootstrap consensus tree inferred from 500 replicates was inferred to represent the evolutionary history of the taxa analyzed (36). The tree was drawn to scale, with branch lengths proportional to the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the maximum composite likelihood method (37) and calibrated with the number of base substitutions per site. Codon positions included here were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated from the dataset using the complete deletion option.

Results

Alignment of COI and 16S rDNA Sequences

A 278-bp fragment of the mitochondrial COI gene and a 289-bp fragment of the mitochondrial 16S rDNA gene were successfully sequenced for all the specimens. No indels (insertion or deletion) was detected. The sequence alignment consists of 324 variable sites on the 278-bp COI region, while only 114 variable sites on the 289-bp 16S rDNA were revealed (Table 2). All the sequences were proofread and aligned in the program MEGALIGN 7.1.0 included in the DNA analysis package DNASTAR and the different bases were bolded.

Phylogenetic Analysis of COI

The phylogeny of sarcophagine flies was clearly separated into four genetic clades (A–D) (Fig. 1). Clade A, which is comprised of nine *S. dux* specimens, was supported by high bootstrap value. Clade B is known as the *albiceps* group as it only contains species *S. albiceps*. Within clade C, the *peregrina* group, two different branches were formed sharing a supporting value of 57%. Four *S. melanura* specimens were united under clade D as the *melanura* group. Clade A and clade B belonged to the genus of *Parasarcophaga*, clustered together with a supporting bootstrap of 60%.

Pairwise divergence between species was calculated and shown in Table 3 (below diagonal). And average variation among all taxon studied was calculated. The number of base substitutions per site from analysis between sequences was shown. The overall average of all specimens was 0.07, interspecific variation varying from 7 to 10%. The maximum and minimum levels of divergence between these four sarcophagid species were similar, ranged from 7 to 11%. The minimum intraspecific values of the four species were all 0%. The maximum and mean values of *S. peregrina* were 8 and 5%, while those of *S. melanura* were 1 and 0%, and for *S. albiceps* were all 1%. No significant intraspecific variation within the *S. dux* samples was observed.

Phylogenetic Analysis of 16S rDNA

At the species level, three distinct congeneric and monophyletic clusters (E–G) were well formed (Fig. 2). The high bootstrap values (≥97%) provide percentage robust support for the monophyly of *S. peregrina, S. albiceps,* and *S. dux*. Within clade H, three *S. melanura* specimens clustered together with a supporting bootstrap of 88% and the specimen from Xining (HM016607) is sister to them with a supporting value of 99%. Clade F and clade G belonged to the genus of *Parasarcophaga* that were not resolved as sister groups, while clade E and clade F clustered together with a supporting bootstrap of 38%.

Pairwise divergence between species was calculated and shown in Table 3 (above diagonal). The overall average of all specimens was 0.03. The levels of interspecific variation varied from 3 to 5%. The maximum and minimum levels of divergence between these four sarcophagid species were close, ranged between 3 and 5%. And the specimens from the same species group exhibited little

TABLE 2—*Variable positions in the 278-bp COI gene fragment (above) and 289-bp 16S rDNA gene fragment (below) alignment of Sarcophagidae specimens.*

Specimen Base No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
COI																							
2	A	A	A	A	A	A	A	A	A	A	**G**	**G**	A	A	T	T	T	T	T	T	T	T	T
3	C	C	C	C	**T**	**T**	C	**T**	**T**	**T**	**T**	**A**	**A**	**T**	**T**	C	C	C	C	C	C	C	C
8	T	T	T	T	**A**	**A**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
17	**T**	T	T	T	T	T	T	T	T	T	T	T	T	T	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
29	A	A	A	A	A	A	A	A	A	A	**T**	**T**	**T**	**T**	A	A	A	A	A	A	A	A	A
32	T	**A**	**A**	**A**	**A**	**A**	**A**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
33	A	A	A	A	A	A	A	A	A	A	**G**	**G**	**G**	**G**	A	A	A	A	A	A	A	A	A
38	T	T	T	T	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
41	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T
44	T	T	**C**	**C**	T	T	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T
45	T	C	C	C	C	C	C	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**
47	A	**T**	**T**	**T**	**T**	**T**	**T**	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
59	C	C	**T**	**T**	C	C	**T**	**T**	**T**	**T**	**T**	C	C	C	**T**	C	C	C	C	C	C	C	C
71	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
74	T	**C**	T	T	**C**	**C**	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T
77	T	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T
80	T	**C**	**C**	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
86	A	A	**C**	**C**	A	A	**C**	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
92	A	A	**T**	**T**	A	A	**T**	**C**	**C**	**C**	**C**	**T**	**T**	**T**	**T**	A	A	A	A	A	A	A	A
95	A	A	A	A	A	A	A	**G**	**G**	**G**	**G**	A	A	A	A	A	A	A	A	A	A	A	A
98	A	A	A	A	A	A	A	**G**	**G**	**G**	**G**	A	A	A	A	A	A	A	A	A	A	A	A
101	T	**A**	T	T	**A**	**A**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
119	T	**A**	**C**	**C**	**A**	**A**	**C**	**A**	**A**	**A**	**A**	T	T	T	T	T	T	T	T	T	T	T	T
122	A	**T**	A	A	**T**	**T**	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
125	T	T	**C**	**C**	T	T	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
128	T	T	T	T	T	T	T	T	T	T	T	**A**	**A**	**A**	**A**	T	T	T	T	T	T	T	T
131	T	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T
137	T	**C**	T	**C**	**C**	**C**	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T
141	T	T	**C**	**C**	T	T	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
146	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T
148	A	A	A	A	A	A	**T**	**T**	**T**	**T**	**T**	A	A	A	A	A	A	A	A	A	A	A	A
149	A	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	A	A	A	A	A	A	A	A	A	A	A	A
153	T	T	**C**	T	T	T	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
165	A	**G**	A	A	**G**	**G**	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
167	A	**T**	A	A	**T**	**T**	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
170	A	A	A	A	A	A	A	**G**	**G**	**G**	**G**	A	A	A	A	A	A	A	A	A	A	A	A
202	T	T	**C**	**C**	T	T	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
218	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T
221	C	C	C	C	C	C	C	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	C	C	C	C	C	C	C	C
224	C	C	C	C	C	C	C	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	C	C	C	C	C	C	C	C
227	T	T	**A**	**A**	T	T	**A**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
233	T	T	T	T	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**
237	T	**C**	T	T	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
239	A	A	A	A	A	A	A	A	A	A	A	A	A	A	**G**	**G**	**G**	**G**	**G**	**G**	**G**	**G**	**G**
242	A	A	**G**	**G**	A	A	**G**	A	A	A	**G**	**T**	**T**	**T**	**T**	A	A	A	A	A	A	A	A
248	A	**G**	A	A	**G**	**G**	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
251	A	**C**	A	A	**C**	**C**	A	A	A	A	A	A	A	A	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**
257	T	T	T	T	T	T	T	T	T	T	**A**	**A**	**A**	**A**	T	T	T	T	T	T	T	T	T
266	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T
269	T	**A**	T	T	**A**	**A**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
272	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T
275	T	**C**	**C**	**C**	**C**	**C**	**C**	**C**	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T
278	A	A	A	A	A	A	A	**T**	A	A	A	**T**	**T**	**T**	**T**	A	A	A	A	A	A	A	A
16S rDNA																							
38	T	T	T	T	T	T	T	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
53	T	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T
70	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	**G**	**G**	**G**	**G**	**G**	**G**	**G**	**G**
72	T	T	T	T	T	T	T	**A**	**A**	**A**	**A**	T	T	T	T	T	T	T	T	T	T	T	T
73	T	T	T	T	T	T	T	**A**	**A**	**A**	**A**	T	T	T	T	T	T	T	T	T	T	T	T
74	T	T	T	T	T	T	T	**G**	**G**	**G**	**G**	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T
89	T	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T
120	T	**C**	**C**	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
124	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T
132	A	**G**	**G**	**G**	**G**	**G**	**G**	A	**G**	**G**	**G**	A	A	A	A	A	A	A	A	A	A	A	A
136	T	T	T	T	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T
137	C	**T**	**T**	**T**	**T**	**T**	**T**	C	C	C	C	**T**	**T**	**T**	**T**	C	C	C	C	C	C	C	C
142	A	A	A	A	A	A	A	A	A	A	A	**T**	**T**	**T**	**T**	A	A	A	A	A	A	A	A
157	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T
160	T	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T
164	C	C	C	C	C	C	C	**T**	**T**	**T**	**T**	C	C	C	C	C	C	C	C	C	C	C	C

Continued

TABLE 2—*Continued.*

Specimen Base No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
203	T	T	T	T	T	T	T	**A**	**A**	**A**	T	T	T	T	T	T	T	T	T	T	T	T	T
204	T	T	T	T	T	T	T	T	T	T	T	T	T	T	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
239	T	T	T	T	T	T	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T
250	T	**C**	**C**	**C**	**C**	**C**	**C**	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
279	T	T	T	T	T	T	T	T	T	T	T	T	T	T	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**

The different bases were bolded. The specimen number in this table is corresponding with Table 1.

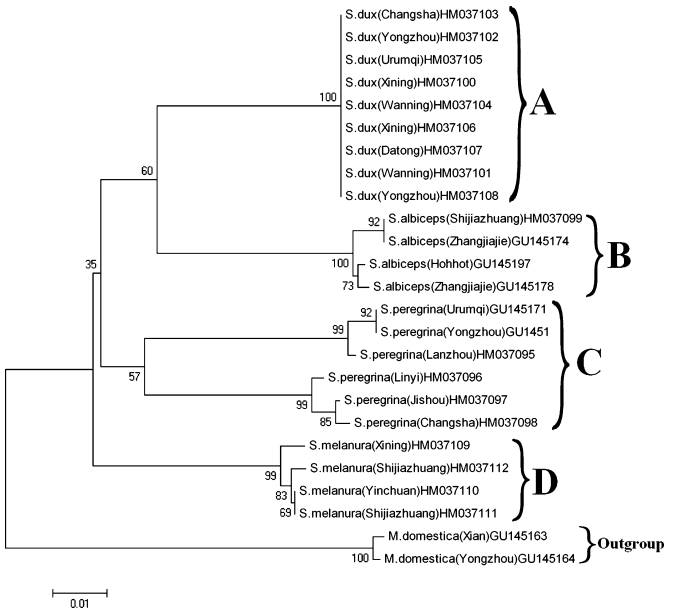

FIG. 1—*Single most parsimonious phylogram of forensic relevant Sarcophagidae species (heuristic search with 500 random step-wise additions) based on a 278-bp region of the COI gene. Numbers on branches indicate the support value. Four major clades were identified as (A) dux group; (B) albiceps group; (C) peregrina group; and (D) melanura group. Neighbor-joining tree of Kimura-two-parameter (K2P) distances for 23 COI gene sequences from four species of Chinese Sarcophagidae. Morphological species identification, voucher ID, and city are given in specimen label. Two* Musca domestica *samples from family Mascidae are included as outgroup. Evolutionary distance divergence scale bar is 0.01.*

divergence based on 16S rDNA, such as *S. peregrina* (0%), *S. melanura* (0–1%), *S. albiceps* (0%), and *S. dux* (0%).

Discussion

Insects can be of significant importance in cases of badly decomposed and unidentified remains and with an undetermined time of death (38). To implement the use of sarcophagids for PMI estimation, a method for easy yet accurate species-level identification at any life stage is required. In this study, we assessed the COI and 16S rDNA sequence as a potential marker for the identification of Sarcophagidae family flies from China. Although the amplicon size and sample size were both small, these data provide an opportunity to evaluate the potential value of COI and 16S rDNA for basic biological studies of these sarcophagid flies. The mtDNA technique can be used as a supplemental means of morphological method in identification of the four Chinese sarcophagid species, as the technology is easier to perform and saves more time for forensic scientists within their routine work.

From Table 2, we can see that much more variable positions on the partial COI analyzed were revealed than that of the partial 16S rDNA, indicating that this selected 16S rDNA region is a highly conserved region. Accordingly, phylogenetic analysis performed based on the COI sequence shows that different branches were formed sharing low supporting values for clade B, clade C, and clade D (Fig. 1). In the genus of *Parasarcophaga*, the two species, *S. dux* and *S. albiceps,* clustered together with a supporting bootstrap of 60%, indicating the efficacy of COI to identify the species from same genus of Sarcophagidae family. In Fig. 2, the monophyletic separation of the four species (clades E–H) in the phylogenetic tree examined by 16S rDNA confirmed the sufficient resolution of the genetic marker. The four species groups of Sarcophagidae were separated and the bootstrap values were all high. However, in the genus of *Parasarcophaga*, the clade F and clade G failed to cluster together directly, 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 COI region. This may be related to the selected region of the 16S rDNA gene, and more sarcophagid species from more locations should be studied in the future.

Genetic species identification is to match the sequence of the evidence item to an authenticated reference DNA sequence. If a sequence of an unknown insect matches a reference sequence, we can conclude that these two taxa are identical or at least belong to the same species complex (3). Once differences occur, information about the intraspecific versus the interspecific variation is necessary to evaluate these differences. In the Table 3 (below diagonal), the interspecific variation between species of different genus was higher than that from same genus, which indicated the efficacy of COI to identify the species from different genus of Sarcophagidae family. Furthermore, the interspecific variation between *S. dux* and *S. albiceps* was larger than 5%, which could distinguish these species of the same genus *Parasarcophaga*. The intraspecific variation within *S. peregrina* samples ranged from 0 to 8%. And the mean level of the intraspecific was 5%, which showed the difference within the *S. peregrina* species. In Table 3 (above diagonal), the interspecific variation examined by 16S rDNA between species was low, and even no significant intraspecific variation was observed within each of the four sarcophagid species. Similar observations have been reported for the flesh flies *Sarcophaga argyrostoma* and *S. crassipalpis* (intraspecific variation 1%, interspecific variation about 3%, respectively) (4) and the blowflies *Calliphora vicina* and *C. vomitoria* (intraspecific variability of less than 1%, interspecific variability of about 5% in the COI) (16).

Detailed systematic studies are required to enable DNA identification of Chinese species encountered in forensic entomology. Seven of the Sarcophagidae are considered to be forensically important in China, including *S. peregrine*, *S. albiceps*, and *S. melanura* (39). To the best of our knowledge, there were no reports on the COI and 16S rDNA sequences analysis of *S. peregrina*, *S. melanura*, *S. albiceps,* and *S. dux* from China. To introduce these two gene sequences as an instrument for forensic entomology, we

TABLE 3—*Pairwise distance matrix of Sarcophagidae 278-bp COI sequences (below diagonal) and the 289-bp 16S rDNA sequences (above diagonal).*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
[1]	–	0.00	0.00	0.00	0.00	0.00	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[2]	0.07	–	0.00	0.00	0.00	0.00	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[3]	0.06	0.01	–	0.00	0.00	0.00	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[4]	0.01	0.08	0.07	–	0.00	0.00	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[5]	0.01	0.08	0.07	0.00	–	0.00	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[6]	0.07	0.00	0.01	0.08	0.08	–	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[7]	0.09	0.08	0.08	0.09	0.09	0.08	–	0.01	0.01	0.01	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
[8]	0.08	0.08	0.08	0.09	0.09	0.08	0.01	–	0.00	0.00	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
[9]	0.08	0.08	0.08	0.09	0.09	0.08	0.01	0.00	–	0.00	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
[10]	0.09	0.08	0.07	0.09	0.09	0.08	0.01	0.00	0.00	–	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
[11]	0.11	0.09	0.09	0.11	0.11	0.09	0.08	0.09	0.09	0.09	–	0.00	0.00	0.00	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[12]	0.11	0.09	0.09	0.11	0.11	0.09	0.08	0.09	0.09	0.09	0.00	–	0.00	0.00	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[13]	0.10	0.08	0.08	0.10	0.10	0.09	0.07	0.08	0.08	0.08	0.01	0.01	–	0.00	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[14]	0.11	0.08	0.08	0.11	0.11	0.09	0.07	0.08	0.08	0.08	0.01	0.01	0.00	–	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
[15]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	–	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[16]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.00	–	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[17]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.00	0.00	–	0.00	0.00	0.00	0.00	0.00	0.00
[18]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.00	0.00	0.00	–	0.00	0.00	0.00	0.00	0.00
[19]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.00	0.00	0.00	0.00	–	0.00	0.00	0.00	0.00
[20]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.00	0.00	0.00	0.00	0.00	–	0.00	0.00	0.00
[21]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.00	0.00	0.00	0.00	0.00	0.00	–	0.00	0.00
[22]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–	0.00
[23]	0.08	0.08	0.08	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	–

The number in this table is corresponding with Table 1. [1]: *Sarcophaga peregrina* (Lanzhou) HM037095; [2]: *S. peregrina* (Jishou) HM037097; [3]: *S. peregrina* (Linyi) HM037096; [4]: *S. peregrina* (Urumqi) GU145171; [5]: *S. peregrina* (Yongzhou) GU145177; [6]: *S. peregrina* (Changsha) HM037098; [7]: *S. melanura* (Xining) HM037109; [8]: *S. melanura* (Yinchuan) HM037110; [9]: *S. melanura* (Shijiazhuang) HM037111; [10]: *S. melanura* (Shijiazhuang) HM037112; [11]: *S. albiceps* (Shijiazhuang) HM037099; [12]: *S. albiceps* (Zhangjiajie) GU145174; [13]: *S. albiceps* (Hohhot) GU145197; [14]: *S. albiceps* (Zhangjiajie) GU145178; [15]: *S. dux* (Datong) HM037107; [16]: *S. dux* (Wanning) HM037104; [17]: *S. dux* (Changsha) HM037103; [18]: *S. dux* (Yongzhou) HM037108; [19]: *S. dux* (Xining) HM037106; [20]: *S. dux* (Urmuqi) HM037105; [21]: *S. dux* (Yongzhou) HM037102; [22]: *S. dux* (Xining) HM037100; [23]: *S. dux* (Wanning) HM037101.

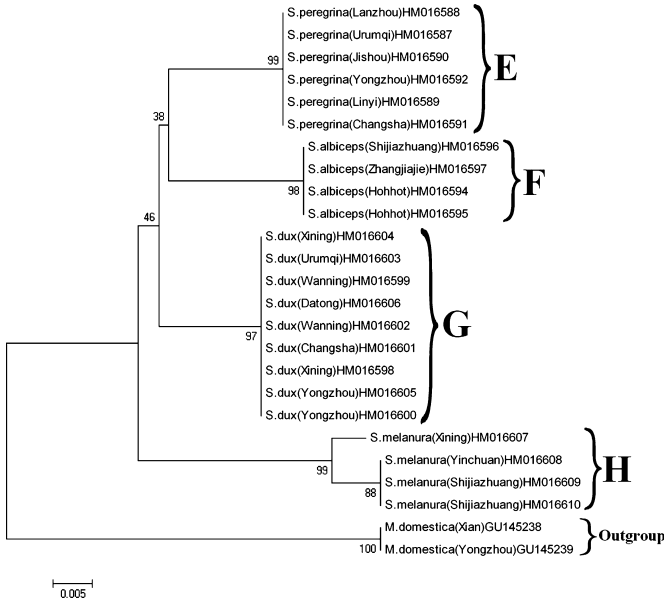

FIG. 2—*Single most parsimonious phylogram of forensic relevant Sarcophagidae species (heuristic search with 500 random step-wise additions) based on a 289-bp region of the 16S rDNA gene. Numbers on branches indicate the support value. Four major clades were identified as (E) peregrina group; (F) albiceps group; (G) dux group; and (H) melanura group. Neighbor-joining tree of Kimura-two-parameter (K2P) distances for 23 16S rDNA gene sequences from four species of Chinese Sarcophagidae. Morphological species identification, voucher ID, and city are given in specimen label. Two* Musca domestica *samples from family Mascidae are included as outgroup. Evolutionary distance divergence scale bar is 0.005.*

assessed the suitability of these genetic markers for identification of the four Chinese sarcophagid species. This is a preliminary study of genetic identification of sarcophagid species, as the amplicon size and sample size were both small; however, the bootstrap support for each group and the level of nucleotide divergence between groups indicating that sarcophagid species identification by COI and 16S rDNA sequences was possible. Future evaluation of more Chinese Sarcophagidae, using different loci, and longer sequences will further improve molecular identification of these forensically important flies. In addition, further sampling to identify additional forensically important Sarcophagidae will help to expand such analyses to all relevant Chinese species.

Conclusion

The 289-bp fragment of the mitochondrial 16S rDNA gene and the 278-bp fragment of the mitochondrial COI gene in this study displayed that DNA-based method can be used as a supplemental means for morphological method in identification of the four Chinese sarcophagid species. As a preliminary study for genetic identification of sarcophagid species, both the sample size and amplicon sizes were small. More samples from different locations and different regions of the COI and 16S rDNA genes need to be studied in the future.

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References

1. Smith JA, Baker NC. Molecular genetic identification of forensically important flies in the UK. Forensic Sci Int Genet 2008;1(1):620–2.

2. Zehner R, Amendt J, Schutt S, Sauer J, Krettek R, Povolny D. Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). Int J Legal Med 2004;118(4):245–7.
3. Amendt J, Krettek R, Zehner R. Forensic entomolog. Naturwissenschaften 2004;91(2):51–65.
4. Wells JD, Pape T, Sperling FAH. DNA-based identification and molecular systematics of forensically important Sarcophagidae (Diptera). J Forensic Sci 2001;46(5):1098–102.
5. Pape T. Catalogue of the Sarcophagidae of the world. Gainesville, FL: Associated Publishers, 1996.
6. Greenberg B. Flies as forensic indicators. J Med Entomol 1991; 28(5):565–77.
7. Pérez-Moreno S, Marcos-García MA, Rojo S. Comparative morphology of early stages of two Mediterran Sarcophaga Meigen, 1826 (Diptera; Sarcophagidae) and a review of the feeding habits of Palaearctic species. Micron 2006;37(2):169–79.
8. Benecke M. Six forensic entomology cases: description and commentary. J Forensic Sci 1998;43(4):797–805.
9. Kamal AS. Comparative study of thirteen species of sarcosaprophagous Calliphoridae and Sarcophagidae (Diptera). Ann Entomol Soc Am 1958;51(3):261–71.
10. Byrd JH, Castner JL, editors. The utility of arthropods in legal investigations. Boca Raton, FL: CRC Press, 2001.
11. Smith KGV. A manual of forensic entomology. London, UK: The Trustees of the British Museum (Natural History), 1986.
12. Kiyoshi S, Masataka T, Yasuhiro A. Species identification of the forensically important flies in Iwate prefecture, Japan based on mitochondrial cytochrome oxidase gene subunit I (COI) sequences. Leg Med 2005;7(3):175–8.
13. Tan SH, Rizman-Idid M, Mohd-Aris E, Kurahashi H, Mohamed Z. DNA-based characterisation and classification of forensically important flesh flies (Diptera: Sarcophagidae) in Malaysia. Forensic Sci Int 2010;3:43–9.
14. Meiklejohn KA, Wallman JF, Dowton M. DNA-based identification of forensically important Australian Sarcophagidae (Diptera). Int J Legal Med 2011;125(1):27–32.
15. Harvey ML, Mansell MW, Villet MH, Dadour IR. Molecular identification of some forensically important blowflies of southern Africa and Australia. Med Vet Entomol 2003;17(4):363–9.
16. Vincent S, Vian J, Carlotti MP. Partial sequencing of the cytochrome oxidase b subunit gene I: a tool for the identification of European species of blow flies for postmortem interval estimation. J Forensic Sci 2000;45(4):820–3.
17. Nelson LA, Wallman JF, Dowton M. Using COI barcodes to identify forensically and medically important blowflies. Med Vet Entomol 2007;21(1):44–52.
18. Ames C, Turner B, Daniel B. The use of mitochondrial cytochrome oxidase I gene (COI) to differentiate two UK blowfly species—Calliphora vicina and Calliphora Vomitoria. Forensic Sci Int 2006;3:179–82.
19. Wallman JF, Donnellan SC. The utility of mitochondrial DNA sequences for the identification of forensically important blow flies (Diptera: Calliphoridae) in southeastern Australia. Forensic Sci Int 2001;2:60–7.
20. Harvey ML, Dadour IR, Gaudieri S. Mitochondrial DNA cytochrome oxidase I gene: potential for distinction between immature stages of some forensically important fly species (Diptera) in western Australia. Forensic Sci Int 2003;3:134–9.
21. Wells JD, Wall R, Stevens JR. Phylogenetic analysis of forensically important Lucilia flies based on cytochrome oxidase I sequence: a cautionary tale for forensic species determination. Int J Legal Med 2007; 121(3):229–33.
22. Wells JD, Lunt N, Villet MH. Recent African derivation of Chrysomya putoria from *C. chloropyga* and mitochondrial DNA paraphyly of cytochrome oxidase subunit one in blowflies of forensic importance. Med Vet Entomol 2004;18(4):445–8.
23. Harvey ML, Gaudieri S, Villet MH, Dadour IR. A global study of forensically significant Calliphorids: implications for identification. Forensic Sci Int 2008;177(1):66–76.
24. Stevens JR, Wall R. Genetic relationships between blowflies (Calliphoridae) of forensic importance. Forensic Sci Int 2001;2:116–23.
25. Stevens JR, Wall R, Wells JD. Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. Insect Mol Biol 2002;11(2):141–8.
26. Han HY. Molecular phylogenetic study of the tribe Trypetini (Diptera: Tephritidae), using mitochondrial 16S ribosomal DNA sequences. Biochem Syst Ecol 2000;28(6):501–13.
27. Han HY, Bruce AM. Molecular phylogenetic study of Tephritidae (Insecta: Diptera) using partial sequences of the mitochondrial 16S ribosomal DNA. Mol Phylogenet Evol 1997;7(1):17–32.
28. Sun XM, Cai JF, Ying BW, Fang YQ, Yun LB, Yuan WA. Identification of sarcosaphagous flies by analyses the sequence of 16S rDNA. J Forensic Sci 2006;22(l):36–8.
29. Lu BL, Wu HY. Classification and identification of important medical insects of China. China: Henan Science and Technology Press, 2003.
30. Fan Z. Key to the common flies of China, 2nd edn. China: Science Press, 1992.
31. Skevington JH, Yeates DK. Phylogeny of the Syrphoidea (Diptera) inferred from mtDNA sequences and morphology with particular reference to classification of the Pipunculidae (Diptera). Mol Phylogenet Evol 2000;16(2):212–24.
32. Sperling FAH, Anderson GS, Hickey DA. A DNA-based approach to the identification of insect specimens used for postmortem interval estimation. J Forensic Sci 1994;39(2):418–27.
33. Wells JD, Sperling FAH. Molecular phylogeny of Chrysomya albiceps and *C. rufifacies*. J Med Entomol 1999;36(3):222–6.
34. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 1993;10(3):512–26.
35. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4(4):406–25.
36. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985;39(4):783–91.
37. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 2004;101(30):11030–5.
38. Greenberg B, Kunich JC, editors. Entomology and the law: flies as forensic indicators. Cambridge, UK: Cambridge University Press, 2002.
39. Min JX. Study of forensically important saprophagous muscoid flies. In: Hou YP, Liu SC, editors. Advances & practices in forensic medicine 2. China: Chengdu Technology Press, 1999;38–45.

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