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Full Length Research Paper

Identification of the forensically important flies (Diptera: Muscidae) based on cytochrome oxidase subunit I (COI) gene in China

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Accurate species identification is a crucial step in forensic entomology, as the insect collected on a corpse can provide useful information for estimation of postmortem interval (PMI). However, morphological distinction may on occasion be impossible to the adult flies and nymphs of the same genus. DNA-based method can be used as a supplemental means of morphological method. In this study, 31 forensically important Muscidae flies were collected from 15 locations in 11 provinces of China, and a 272 base pair region of the mitochondrial cytochrome oxidase subunit I (COI) gene was sequenced. The monophyletic branches of the phylogenetic tree revealed that this marker is suitable for discrimination between these five species of four genera of Muscidae. The genetic variations found on COI can be applied not only to identify the forensically important species, but also to understand the taxonomic positions of the sarcophagine species. In addition, this research will be instrumental for implementation of the Chinese Muscidae database.

Key words: Forensic science, forensic entomology, Muscidae, cytochrome oxidase subunit I (COI), species identification.

INTRODUCTION

Muscidae found on human corpses is important for the estimation of the postmortem interval and other questions of forensic relevance (Byrd and Castner, 2010; Cai, 2011). Many Muscidae species are the first to locate and oviposit onto corpses, and are among the dominant species found on corpses (Linhares, 1981; Smith, 1986). *Musca* is distributed worldwide and commonly known as house flies or stable flies due to their synanthropy (Omar et al., 2003). According to Nurita et al. (2008), the *Musca domestica* rank first in the prevalence in all their studies, which is similar to the previous study showing the appearance of *M. domestica* at fisheries, slaughter-houses, garbage disposal sites, vegetable farms, market places and poultry farms (Sulaiman et al., 1988).

Precise species identification of every insect sample collected from criminal scenes play an essential role in the accurate estimation of postmortem interval (PMI), especially when information on the postmortem phenomena is not available (Amendt et al., 2004). However, their morphological similarity poses a great challenge for forensic entomologists, and up to now, it is still difficult to diagnostic certain groups of Muscidae family at their egg, pupa and larval stages by using morphological criteria (Cai, 2011). Furthermore, time- consuming rearing of the larvae to adults for identification may delay the criminal

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investigation or cause significant problems when rearing fails (Amendt et al., 2004). DNA-based method, as a supplemental means of morphological method, can provide a quick and reliable species determination and is relatively insensitive to the preservation methods.

Meanwhile, DNA-based identification can be carried out on any lifecycle stage without further rearing and on dead, preserved or live samples (Malgorn and Coquoz, 1999). Mitochondrial DNA (mtDNA) has the advantages of easy isolation, higher copy number than its nuclear counterparts, supposed high mutation regions, and conserved sequence and structure across taxa, making discrimination between species and even sub-species possible (Harvey et al., 2008). Although 16S ribosomal RNA (16S rRNA) had been used to solve species identification of forensically important flies, partial 16S rDNA region to identify the species from same genus was not as efficient as that of the partial cytochrome oxidase subunits one (COI) region (Guo et al., 2011). COI gene of different lengths and regions have been studied, and several short fragments were proven to have sufficient discrimination power, (Harvey et al., 2003; Zehner et al., 2004; Saigusa et al., 2005; Ames et al., 2006; Desmyter and Gosselin, 2009) which makes it in particular suitable for forensic applications.

The composition of each species has its own local characteristics. According to Grassberger and Frank (2004), no global generalizations may be made in precisely estimating PMI or the time of death. Entomological evidence must be evaluated on a regional scale and the creation of local databases with referred ecological data for insect identification is strongly recommended (Alessandrini et al., 2008). This study evaluates the suitability of the 272 bp COI region for identification of Muscidae species under experimental conditions prior to application in Chinese criminal investigations. The 272 bp COI region of 31 Muscidae flies was sequenced and deposited in GenBank to expand local databases.

MATERIALS AND METHODS

Specimens

Seven specimens of *M. domestica* (Linnaeus, 1758), five specimens of *Ophyra spinigera* (Stein, 1910), six specimens of *Ophyra chalcogaster* (Wiedemann, 1824), nine specimens of *Muscina stabulans* (Fall, 1823) and four specimens of *Stomoxys calcitrans* (*Linnaeus*, 1758) were obtained during the months of June to September in China from 2007 to 2010 (Figure 1). Two dried adult specimens of *Silpha carinata* (Herbst, 1783) (Coleoptera) were obtained in the year 2009. Each specimen was assigned a unique code. Data collections for all specimens used in this study are 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 -20°C. All samples were identified using morphological keys with the assistance of entomologists from Hunan Agricultural University (Lu and Wu, 2003; Xu and Zhao, 1996). The specimens were observed under a stereomicroscope to identify the species based on regional hair and colour, body pigmentation, bristle length and/or the shape of the genitalia.

DNA extraction

The mtDNA of all samples were extracted using the cetyltrimethyl-ammonium bromide (CTAB) method (Guo et al., 2010). To avoid possible contamination of fly DNA with DNA from ingested protein and gut parasites of eggs, only the thoracic muscle of each insect was used as a source of DNA, while the remains of each specimen were stored in 1.5 ml Eppendorf (EP) tube at -20°C to check the identity and for accumulation of samples. These remains with unique codes were preserved in our laboratory permanently (Table 1).

Polymerase chain reaction (PCR) protocol

A portion of 272 bp fragment of the mitochondrial COI gene was amplified and sequenced by using forward primer (5′-CAGATCGAAATTTAAATAC-TTC-3′) and reverse primers (5′-GTATCAACATCTATTCCTAC-3′); detail of the primers were described by Liu et al. (2011). The PCR reaction volume was 25 µL, containing 1 to 5 µL (20 to 40 ng) of template DNA, 12.5 µL 2× GoTaq® Green Master Mix (Promega, Madison, WI, USA), 4 µL dNTP (1 mmol/ml, 1.0 U Taq polymerase, 2.5 µL 10X buffer (Mg^{2+} 1.5 mmol/L), 0.25 to 2.5 µL of each primer (10 µM). Nuclease-Free Water (Promerga, Madison, WI, USA) was added to a total volume of 25 µL. PCR amplifications were performed in a thermocycler (Perkin-Elmer9600) and programmed with the following parameters: initial step at 94°C for 3 min then continued for 30 cycles each at 94°C for 30 s; 50°C for 30 s; 72°C for 30 s. An elongation of PCR products by 72°C for 5 min completed the reaction.

Sequencing

Vertical non-denaturing polyacrylamide gel electrophoresis was used to isolate PCR products, and 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 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 were 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 BLAST function.

Also, to determine whether they were of mitochondrial origin or represented paralogous sequences in the nucleus, the sequences were tested using MEGA4 (Tamura et al., 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.31417754 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The

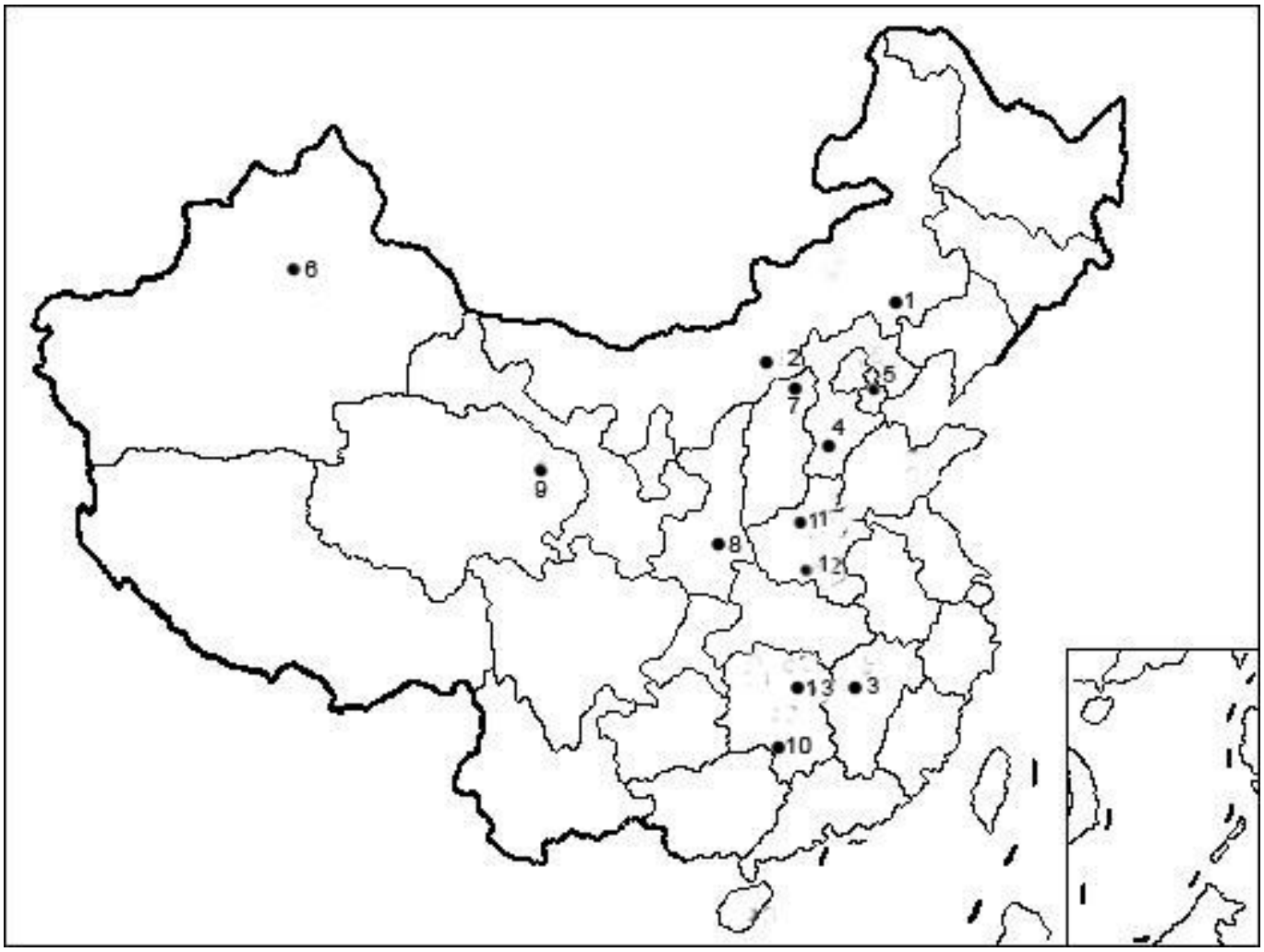

Figure 1. Map showing collection locations of sarcosaphagous Muscidae in China. 1, Baotou InnerMongolia; 2, Hohhot InnerMongolia; 3, Yichun Jiangxi; 4, Shijiazhuang Hebei; 5, Tianjin; 6, Urumqi Xinjiang; 7, Datong Shanxi; 8, Xi'an Shanxi; 9, Qinghai Xining; 10, Yongzhou Hunan; 11, Xinxiang Henan; 12, Nanyang Henan; 13, Changsha Hunan.

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., 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 272 positions in the final dataset. Two specimens belonging to Coleoptera were used as outgroup for phylogenetic analyses (Table 1).

RESULTS AND DISCUSSION

All the collected specimens from the Muscidae family were divided into five species belonging to four genus: (1) *Muscina*: *M. stabulans* (Fall, 1823) (nine specimens); (2) *Musca*: *M. domestic*a (Linnaeus,1758) (seven specimens); (3) *Ophyra*: *H. spinigera* (Stein) (five specimens); *H. chalcogaster* (Wiedemann) (six specimens); (4) *Stomoxys Geoffroy*: *S. calcitrans* (Linnaeus) (four specimens). Adults of *M. domestica* often visited the carcasses at an early stage of decomposition, such as bloat and active stage, as had been reported by Sharanowski et al. (2008). The absence of larvae of *M. domestica* was also reported in a previous study (Heo et al., 2008). The adults and larvae of *O. spinigera* and *O. chalcogaster* visited the carcasses at advance-decay stage, which was also observed by other studies (Heo et al., 2008; Wang et al., 2008).

Moreover, the mtDNA of all specimens were successfully extracted and sequenced. We assessed the COI sequence as a potential marker for the identification of Muscidae family flies from China. The intra versus interspecific variation also marked the clear threshold levels for species identification based on this COI region. Since adults and immature have identical genotypes, and species identification from immature Diptera by the DNA

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

Number	Species	Accession number	ID of specimen	Collected location	Date
1		GU269971	DM001A	Shijiazhuang, Hebei [114:26E,38:03N]	2009.8
2		HM016703	DM003B	Yichun, Jiangxi [114:38E, 27:47N]	2010.8
3		HM016701	DM101C	Datong, Shangxi [113:13E 40:07N]i	2007.9
4		GU269972	DM031A	Urumqi, Xinjiang [87:36E, 43:46N]	2007.9
5	*M. stabulans*	HM016700	DM022B	Xining, Qinghai [101:49E, 36:37N]	2007.9
6		HM016698	DM201A	Changsha, Hunan [112:59E 28:12N]	2007.8
7		HM016702	DM089A	Yichun, Jiangxi [114:38E, 27:47N]	2007.8
8		HM016699	DM061C	Urumqi, Xinjiang [87:36E, 43:46N]	2008.8
9		GU269970	DM007B	Urumqi, Xinjiang [87:36E, 43:46N]	2008.8
10		GU269968	DM009A	Yongzhou, Hunan [111:61E, 26:42N]	2008.8
11		GU269966	DM078A	Yichun, Jiangxi [114:38E, 27:47N]	2009.7
12		GQ118410	DM077A	Baotou, InnerMongolia [110:00E 40:35N]	2009.7
13	*M. domestica*	GU269967	DM129B	Xi'an, Shangxi [108:91E, 34:23 N]	2009.7
14		GU269969	DM123C	Baotou, InnerMongolia [110:00E 40:35N]	2010.8
15		HM016697	DM071A	Xinxiang, Henan [113.52E,35.18N]	2009.8
16		GU269964	DM077A	Nanyang, Henan [112.21E,34.40N]	2009.8
17		HM016708	DM081B	Nanning, Guangxi [108.3E,22.8N]	2009.8
18		GU269978	DM091A	Changsha, Hunan [112:59E 28:12N]	2009.8
19	*O. spinigera*	HM016709	DM032C	Changsha, Hunan [112:59E 28:12N]	2009.9
20		HM016710	DM006A	Tianjin, Tianjin [117.2E ,39.13N]	2010.9
21		GU269976	DM121B	Xi'an, Shangxi [108:91E, 34:23 N]	2009.9
22		HM016704	DM157A	Urumqi, Xinjiang [87:36E, 43:46N]	2008.9
23		GU269975	DM190C	Yongzhou, Hunan [111:61E, 26:42N]	2008.9
24	*O. chalcogaster*	HM016706	DM004B	Hohhot, InnerMongolia [111:38E,40:48N]	2008.9
25		GU269974	DM178A	Hohhot, InnerMongolia [111:38E,40:48N]	2009.8
26		HM016705	DM008A	Yichun, Jiangxi [114:38E, 27:47N]	2009.8
27		HM016707	DM091C	Yongzhou, Hunan [111:61E, 26:42N]	2008.8
28		GU269973	DM093A	Xining, Qinghai [101:49E, 36:37N]	2007.9
29	*S. calcitran*	HM016712	DM076B	Xining, Qinghai [101:49E, 36:37N]	2007.9
30		HM016711	DM069A	Yichun, Jiangxi [114:38E, 27:47N]	2009.8
31		HM016713	DM037C	Yichun,J iangxi [114:38E, 27:47N]	2009.8
	Outgroup				
32	*S. carinata*	GU269997	CS038A	Xi'an, Shangxi [108:91E, 34:23 N]	2007.9
33		GU269999	CS070A	Shijiazhuang, Hebei [114:26E, 38:03N]	2009.8

sequences was simple and time-saving since there is no need to wait for adult emergence or knowledge of morphological keys (Tourle et al., 2009), molecular-based species identification method would be as a practically useful approach for further forensic caseworks in China. Furthermore, once we had one specimen confirmed to a species, a further search for more diagnostic morphological characters on larvae and adults individually will be probable.

There were 272 aligned sites for the 31 COI sequences analysis (Figure 2). As outgroup, the two *S. carinata* samples belonging to Coleoptera clustered together and were clearly separated from the Muscidae mitotypes (Figure 2). At the species level, each species formed its own distinct conspecific and monophyletic cluster with high bootstrap values. The high support for the congeneric grouping of species illustrated the potential of the COI in interspecific distinction. All the specimens were classified into five main groups with high bootstrap support. Different clades were formed within each of the

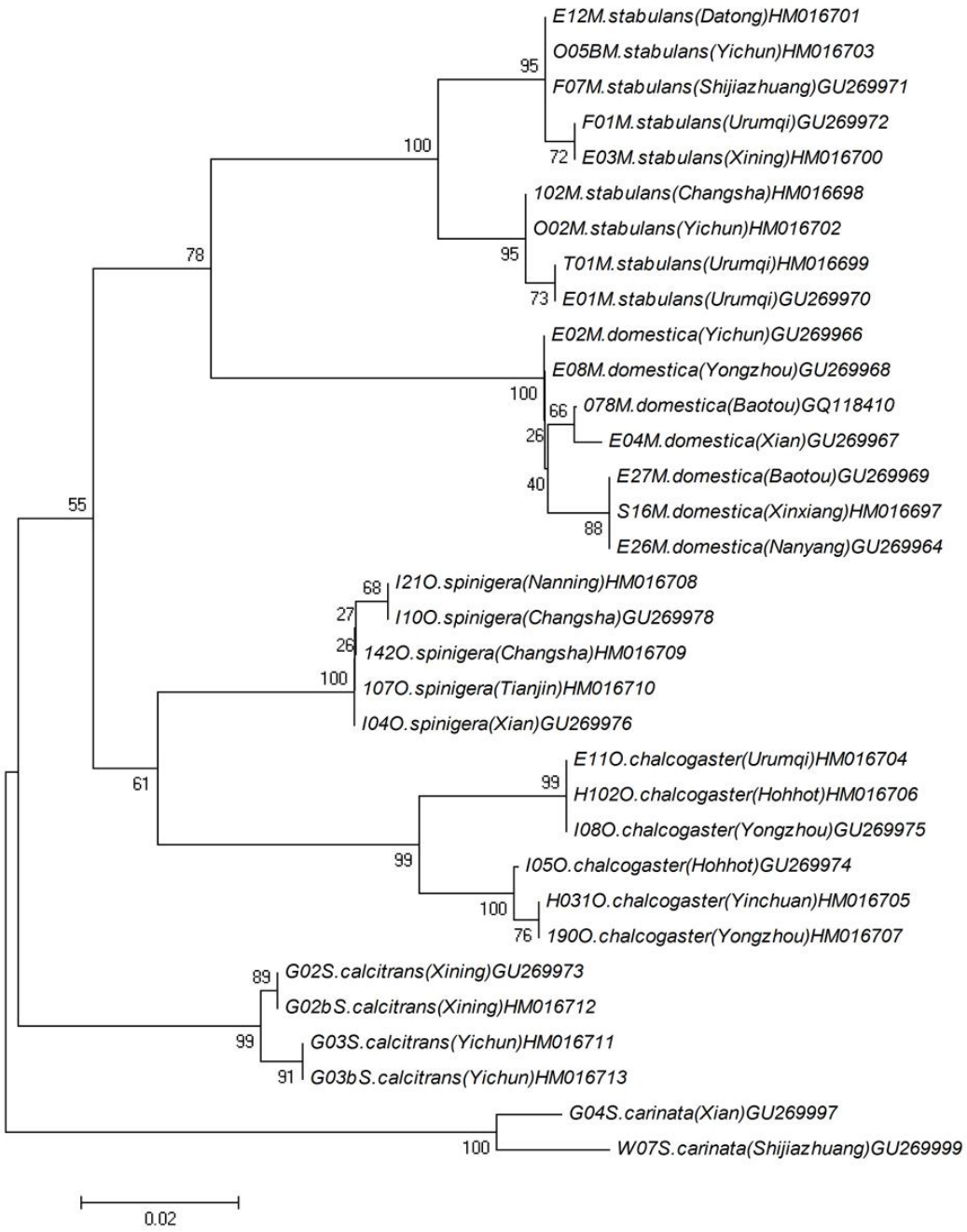

Figure 2. Neighbor-joining (NJ) tree of Kimura-two-parameter (K2P) distances for 31 cytochrome oxidase subunit I (COI) gene sequences from five species of Chinese Muscidae. Morphological species identification, voucher ID and City are given in specimen label. Numbers on branches indicate the support value. Two specimens of *Silpha carinata* (Herbst, 1783) from family Coleoptera were included as outgroup. Evolutionary distance divergence scale bar is 0.01.

species. In the genus of *Ophyra*, the two species (*O. spinigera* and *O. chalcogaster*) clustered together with a supporting bootstrap of 61%, indicating the efficacy of COI to identify the species from same genus of Muscidae family. Furthermore, the *Muscina* and *Musca* were unexpectedly clustered together with a supporting bootstrap of 78%, indicating that more specimens from more locations should be studied in the future.

Nevertheless, no variation was detected between the individuals from different regions for some species (intraspecific variation is 0.0%), although COI is a relatively fast-evolving gene (Table 2), which indicates that these species might diffuse recently, or it may be due to the relatively short COI region (272 bp) analyzed

Table 2. Calculated intraspecific and interspecific variations expressed as a percentage of the 272 bp COI data.

S/N	Species	Number of species	Range and mean	Interspecific variations mean				
1	*M. domestica*	7	0-10 (0.7)	-				
2	*M. stabulans*	9	0-3 (1.7)	0.90	-			
3	*O. chalcogaster*	6	0-8 (2.0)	1.30	0.11	-		
4	*O. spinigera*	5	0-10 (0.2)	0.90	0.10	0.08	-	
5	*S. calcitrans*	4	0-10 (0.5)	1.10	0.13	0.10	0.07	-

herein. Table 2 shows the divergence values between every two species within Muscidae family. The divergence range between the species *M. domestica* and *O. chalcogaster* was the highest at 12 to 13%, while others varied at 3 to13%. Table 2 shows that the level of COI sequence nucleotide divergence and the group *O. chalcogaster* had the maximum intraspecific variation, which was 2.0%. Similar observations were reported for the flesh flies *Sarcophaga argyrostoma* and *Sarcophaga crassipalpis* (intraspecific variation 1%, interspecific variation about 3%, respectively) (Wells et al., 2001) and the blow flies *Calliphora vicina* and *Calliphora vomitoria* (intraspecific variability of less than 1%, interspecific variability of about 5% in the COI) (Vincent et al., 2000). Pair-wise divergence between species was also calculated and is shown in Table 2. The maximum level of interspecific variation was found in species *M. domestica* and *O. chalcogaster*, which indicate the efficacy of COI sequence to identify the species from different genus of Muscidae family.

The local databases (plus the referred ecological data) are also strongly recommended to set up to reduce the incidence of geographic variations on the species identification. Some researches supposed that small fragment of DNA may fail to produce an accurate representation of the total genetic variability in that gene (wells et al., 2001). However, many projects, such as Barcodes of Life (http://barcoding.si.edu/DNABarCoding.htm) are still opting to use only relatively short DNA fragments. Although longer fragments may minimize stochastic variation across taxa and be more likely to reflect broader patterns of nucleotide divergence (Roe and Sperling, 2007), shorter fragments have many advantages such as being quick, easy and economical.

Extensive application of long mtDNA segments for species identification cannot always be achieved due to constraints in time and money. Of course, relying upon a single short DNA fragment for identification is dangerous as the results may be misleading (Wells and Stevens, 2008). Thus, various regions of DNA should be studied to find the best region with truly diagnostic nucleotide changes in future studies.

Conclusion

The 272 bp region of the COI sequence in our study revealed the potential of mtDNA for use as a discriminatory tool in forensic investigation, and also show that mitochondrial nucleotide sequences can offer a means of identifying forensically important Chinese insects. As a preliminary study of genetic identification of Muscidae species, both the sample size and amplicon sizes were small. More samples from different locations and different regions of the COI gene need to be studied in the future.

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