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The availability of 16S rRNA for the identification of forensically important flies (Diptera: Muscidae) in China

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Abstract. Many species of Muscidae are cosmopolitan synanthropic insects. It is frequently found on dead bodies after death, so an accurate identification of each species is necessary for the correct estimation of the postmortem interval (PMI). Identification species of Muscidae is traditionally performed through their morphological features. However, the morphological similarity of Muscidae in the level of species usually poses a challenge for forensic entomologists. Therefore a molecular method used 16S rRNA sequence on mitochondrial DNA was established for species identification. In this study, a 289 base pair region of mitochondrial DNA (mtDNA) coding for 16S rRNA was investigated for identification of the following forensically important species of Muscidae. The specimens were from four genera, including 18 *Musca domestica* (Linnaeus, 1758), 2 *Ophyra spinigera* (Stein, 1910), 2 *Ophyra chalcogaster* (Wiedemann, 1824), 1 *Morellia hortorum* (Fallén, 1817), and 1 *Fannia canicularis* (Linnaeus, 1761). 16S rDNA region allowed the identification of these species of Muscidae, indicating the possibility of separation congeneric species with molecular method.

INTRODUCTION

Forensic entomology is a branch of forensic science which focuses on the use of insects and other arthropods in forensic cases. It has increasingly attracted attention worldwide. Forensic entomologists often solve the problems about the cause and place of death and the estimation of postmortem interval (PMI) by analyzing the development or succession of arthropods on corpse (Keh, 1985; Matuszewski *et al.*, 2008).

Muscidae is a family of flies belongs to the superfamily Muscoidea. They are worldwide in distribution and are commonly known as house flies or stable flies due to their synanthropy. Many species of Muscidae are even coprophilic (Linhares, 1981). Some data suggest that flies of Muscidae family belonging to a complex of species are attracted to animal carcasses, and the first larval stages are necrophagous (Smith, 1986). As the occurrence of many species from Muscidae was observed throughout the world, more and more forensic entomologists have concentrated on this species to find relative information of forensic cases.

Musca is an important genus in the Muscidae family. And *Musca domestica* is one of the most predominant synanthropic fly species in this genus. It is strongly associated with urban areas throughout the

world (Greenberg, 1973). The multiplication of this fly begins at spring with the increase in temperature and the number of species reaches a peak at the beginning of the autumn. At the subtropical area, the species of *M. domestica* can reproduce all the year round. In Nurita *et al.* (2008), *M. domestica* ranked first in prevalence in all their three studies, which is similar to a previous study that showed the presence of *M. domestica* at fisheries, slaughterhouses, garbage disposal sites, vegetable farms, market places and poultry farms (Sulaiman *et al.*, 1988). Besides, *M. domestica* is one of the most commonly encountered insects species used in forensic investigation. Heo *et al.* (2008) firstly reported the case of *M. domestica* eggs found on a pig carcass, indicating the *M. domestica* maybe an early visitor on fresh pig carcass. They also explained the non-finding of *M. domestica* stages for the presence of predators. Also, some other researches showed the succession of *M. domestica* (Tabor *et al.*, 2005; Wang *et al.*, 2008). Particularly, some researchers reported the predominant presence of *M. domestica* in the summer, which indicated the influence of season for the presence of *M. domestica* (Arnaldos *et al.*, 2005). Because of these characteristics of *M. domestica*, more and more forensic entomologists have concentrated on this species.

Other genus of Muscidae flies such as *Ophyra* is also important in forensic investigation. Flies in this genus are more commonly found in urban settings, and they prefer fermenting areas so they often are found in vegetation, faeces or decomposing carcasses. In a study by Omar *et al.* (1994) larvae and adults of *Ophyra spinigera* were observed on monkey carcass as major colonizer, in the late or highly decomposed stage, while Heo *et al.* (2008) observed the larvae of *O. spinigera* on the pig carcass at the advance-decay stage of decomposition. Furthermore, Wang *et al.* (2008) found numerous larvae of *O. spinigera* appear on the pig carcasses. While the active time of adults and larvae were earlier in summer and later in autumn, and adults and larvae of *O. spinigera* nearly disappeared in winter. They also found the succession of *Ophyra chalcogaster* on pig carcasses. Besides, the muscid genera *Morellia* and *Fannia* are usually considered important in forensic investigation. Sharanowski *et al.* (2008) observed the occurrence of adults from *Morellia* genus in spring and summer from sunlit and shaded pig carrion, and adults of *Fannia* from sunnit pig carrion.

However, sometimes the identification of sarcophagous insects including the species from the Muscidae can be puzzling because of the similar morphological markers, and it can be difficult or even impossible to identify the immature stages of many species (Benecke & Wells, 2001). For example, morphological diversity among *M. domestica* has led to the recognition of intraspecific taxonomic status to different forms (Marquez & Krafsur, 2002).

Under such circumstances, mitochondrial DNA (mtDNA) analysis can be used to solve these problems and mtDNA is clearly advantageous in forensic entomology studies. It has a higher mutation rate than nuclear DNA and, therefore, an increased chance of generating species-specific markers. In previous studies, the cytochrome oxidase I gene (COI) and cytochrome oxidase II gene (COII) of mtDNA were used more frequently (Wells & Williams, 2007; Ying *et al.*, 2007). And some studies have indicated that the sequence of 16S rRNA accumulates mutations more rapidly than the nuclear rDNA genes and can infer relationships beneath the family level within insects (Simon *et al.*, 1994). This study has explored the utility of the independent 16S rRNA sequences to the identification of the Muscidae species as well as the morphological data.

MATERIALS AND METHODS

Specimens

Eighteen specimens of *M. domestica*, 2 specimens of *O. spinigera*, 2 specimens of *O. chalcogaster*, 1 specimen of *M.*

hortorum and 1 specimen of *F. canicularis* were obtained from 13 different locations in China since 2006. All of the collections were performed in summer from July to September. Collection data for all specimens of the above species included in the study are given in Table 1. Two kinds of baits were used: rat carcasses and pig carcasses. The animals' 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 -20ºC. All specimens were identified by an expert entomologist on morphology.

DNA extraction

The mtDNA of all samples were extracted using the improvement in grinding tissue during extracting DNA from small insects (An *et al*., 2003) with modification. To avoid possible contamination of foreign DNA from ingested protein and gut parasites of eggs, only the thoracic muscle of each insect was used as a source of DNA.

PCR primers

All of the 16S rRNA sequences were aligned using the sequence alignment programme DNASTAR (Megalign version 7.1.0). 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 programme Primer Premier 5.0. 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 289bp fragment of the mitochondrial 16sRNA gene was amplified and sequenced by using forward primer (5'-CGCTGTTATCCCTAAGGTAA-3') and reverse primer (5'-CTGGTATGAAAGGTTT GACG -3').

PCR conditions

The PCR reaction volume was 25µl, containing 1-5µl DNA, 12.5µl GoTaq® Green Master Mix (4µl dNTP (1mmol/ml), 1.0µl Taq polymerase, 2.5µl 10×buffer (Mg2+1.5mmol/l)), 0.25-2.5µl upstream primer (10µM), 0.25-2.5µl downstream primer (10µM), 25µl Nuclease-Free Water.

PCR amplifications were performed in a thermocycler (Perkin-Elmer9600), with initial denaturing for 2 min at 95ºC, followed by 38 cycles of 94ºC for 1 minute, 48ºC for 1 minute and 72ºC for 2 minutes.

Sequencing

After purification of the PCR products with QIA-quick columns cycle, sequencing was performed on both forward and reverse strands using ABI PRISM 3730. And the sequencing agent was BigDye terminator v3.1. Sequence chromatograms were edited and discrepancies between forward and reverse sequences resolved using Sequence Navigator (v1.01, Applied Biosystems), and the resultant sequences were aligned using the programme ClustalW (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html).

Phylogenetic analysis

A total of 289 aligned sites for the 24 fragments of the mitochondrial 16S rRNA sequences were included in the analyses. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree was constructed by the software of MEGA 4.0 (Tamura & Nei, 1993). And we analyzed whether the sequences were of mitochondrial origin or represented paralogous sequences resident in the nucleus using MEGA 4.0. We also compared the base composition of the individual sequences since nuclear paralogues can have divergent base compositions relative to mitochondrial genes.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

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

Species	Code No.	Location (Latitude (°), Longitude (°))	Presevation Method	Accession Number
Musca domestica (Linnaeus,1758)	a	Chifeng, Inner Mongolia (42.26, 18.89)	70% ethano	FJ763661
	b	Changsha, Hunan (28.23, 112.94)	70% ethano	GQ118387
	c	Yongzhou, Hunan (26.42, 111.61)	70% ethano	FJ763685
	d	NanYang, Henan (32.99, 112.53)	70% ethano	FJ763679
	e	Linyi, Shandong (35.05, 118.34)	70% ethano	FJ763695
	f	Datong, Shanxi (40.08, 113.31)	70% ethano	FJ763654
	g	Jianghua, Hunan (24.97, 111.79)	Dried	FJ763691
	h	Baotou, Inner Mongolia (40.66, 109.84)	70% ethano	FJ763665
	i	Yinchuan, Ningxia (38.46, 106.91)	70% ethano	GU145237
	j	Xi'n, Shannxi (34.23, 108.91)	70% ethano	GU145238
	k	Yongzhou, Hunan (26.42, 111.61)s	Dried	GU145239
	l	Yongzhou, Hunan (26.42, 111.61)	Dried	GQ396693
	m	Shijiazhuang, Hebei (38.04, 114.51)	70% ethano	FJ763657
	n	Fuzhou, Fujian (25.18, 118.08)	70% ethano	GQ118376
	o	Fuzhou, Fujian (25.18, 118.08)	70% ethano	GQ396683
	p	Xiangxiang, Hunan (27.44, 112.31)	70% ethano	FJ763710
	q	Jishou, Hunan (28.30, 109.71)	70% ethano	FJ763711
	r	Jishou, Hunan (28.30, 109.71)	70% ethano	CQ396696
Morellia hortorum (Fallén, 1817)	s	Chifeng, Inner Mongolia (42.26, 118.89)	70% ethano	GQ118375
Ophyra chalcogaster (Wiedemann, 1830)	t	Hohhot, Inner Mongolia (40.49, 111.48)	70% ethano	GU145258
	u	Yongzhou, Hunan (26.42, 111.61)	70% ethano	GU145260
Ophyra spinigera (Stein, 1910)	v	Changsha, Hunan (28.23, 112.94)	Dried	GU145261
	w	Nanning, Guangxi (22.82, 108.37)	70% ethano	FJ763660
Fannia canicularis (Linnaeus, 1761)	y	Beijing (39.92, 116.46)	70% ethano	GQ118368

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and were in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 289 positions in the final dataset.

RESULTS

Alignment of 16S rRNA sequences

289 bp fragment of the mitochondrial 16S rRNA gene was successfully sequenced for all the specimens. And the alignment of all specimens considered in this study lacked any insertion or deletion and revealed 68 variable positions on 289 analysed (Figure 1). All 16S rRNA sequences were aligned through the programme DNASTAR (Megalign version 7.1.0), before the final adjustments were made by eyes. The same bases were marked dark, while the different ones were marked light.

Phylogenetic analysis

A total of 289 aligned sites for the 24 fragment of the mitochondrial 16S rRNA sequences were included in the analyses (Figure 2).

In the Figure 2, the neighbor-joining (NJ) tree was constructed using MEGA, the consensus tree was computed, and the bootstrapped version was resulted in the same tree. The optimal tree with the sum of branch length (0.41058751) was shown. It was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. In the genus of *Musca* (*M. domestica*), 2 specimens from Fuzhou (GQ118376, GQ396683) and 2 specimens from Jishou (FJ763711, GQ396696) formed two single clades with the support value of 100% respectively, which indicated the close genetic relationship between these specimens. The low support value of 4% between the specimens from Chifeng and Linyi showed their difference based on geographical reason.

In the genus of *Ophyra* (*O. spinigera* and *O. chalcogaster*), 4 specimens were grouped together, and 2 specimens of *O. spinigera* and 2 specimens of *O. chalcogaster* formed two single clades with the support value of 88% respectively. Furthermore, 1 specimen of *M. hortorum* and 1 specimen of *F. canicularis* were grouped together with the support value of 40%.

Indeed, this tree provided the strong support for the integrity of the species from Muscidae family. The analysis also indicated intraspecific variation of *M. domestica*, *O. spinigera* and *O. chalcogaster* across wide geographical areas.

Interspecific and intraspecific variation

All results were based on the pairwise analysis of the 24 sequences. Analyses were conducted using the Maximum Composite Likelihood method in MEGA 4.0 (Tamura *et al*., 2004; 2007). Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Pairwise divergence between species was calculated and are showed in Table 2. And variation among all individuals of the species 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

In Table 2, levels of interspecific variation varied from 0% to 16%. The maximum and minimum levels of divergence between the *F. canicularis* and *M. domestica* were 15% and 8%, that of *F. canicularis* and *O. chalcogaster* were 10% and 9%, that of *F. canicularis* and *O. spinigera* were 6% and 8%, that of *M. domestica* and *O. chalcogaster* were 15% and 7%, that of *M. domestica* and *O. spinigera* were 15% and 8%, that of *M. domestica* and *M. hortorum*

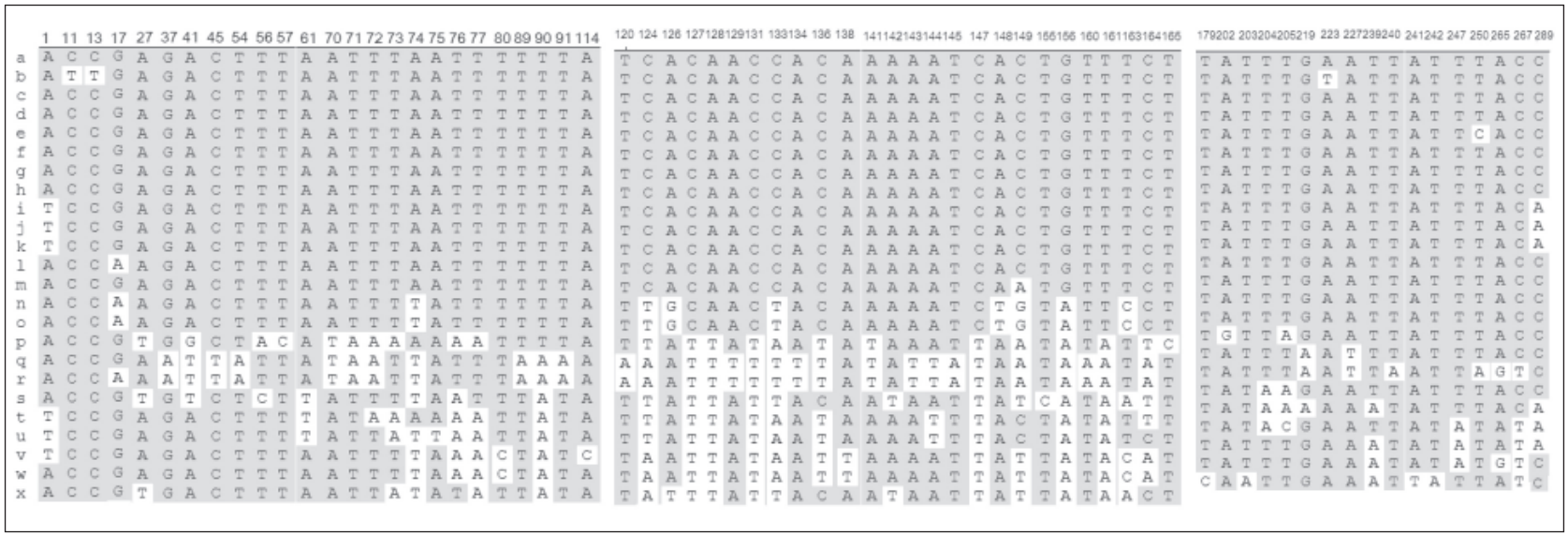

Figure 1. Variable positions in the 289 bp 16S rRNA gene fragment alignment of Muscidae specimens

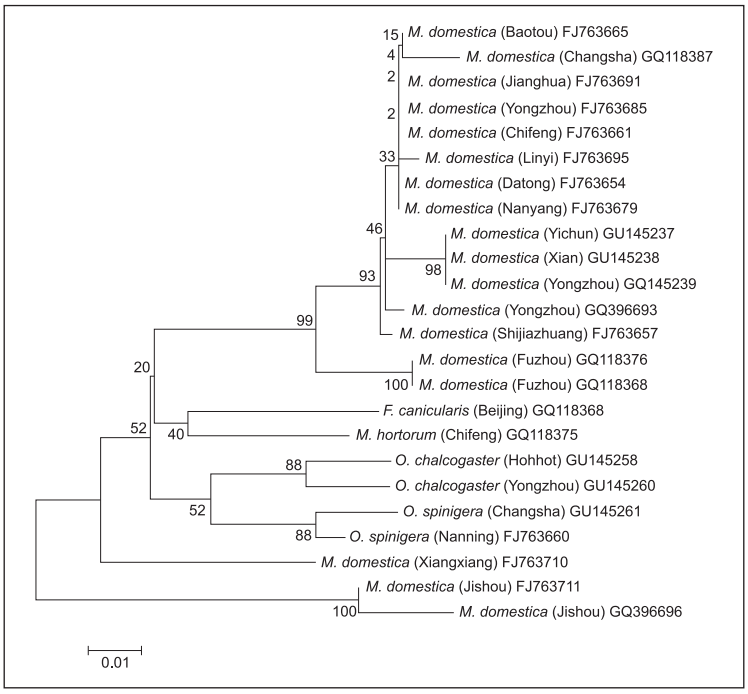

Figure 2. Single most parsimonious phylogram of forensic relevant Muscidae species (heuristic search with 1000 random step-wise additions) based on a 289 bp region of the 16S rRNA gene. Numbers on branches indicate the support value

Table 2. Pairwise distance matrix of Chrysomyinae 289bp 16S rDNA sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
[1]	-																							
[2]	0.09	-																						
[3]	0.10	0.01	-																					
[4]	0.09	0.00	0.01	-																				
[5]	0.09	0.00	0.01	0.00	-																			
[6]	0.08	0.03	0.04	0.03	0.03	-																		
[7]	0.08	0.03	0.04	0.03	0.03	0.00	-																	
[8]	0.09	0.00	0.01	0.00	0.00	0.03	0.03	-																
[9]	0.13	0.12	0.14	0.12	0.12	0.13	0.13	0.12	-															
[10]	0.15	0.15	0.16	0.15	0.15	0.14	0.14	0.15	0.02	-														
[11]	0.09	0.00	0.01	0.00	0.00	0.04	0.04	0.00	0.13	0.15	-													
[12]	0.09	0.00	0.01	0.00	0.00	0.03	0.03	0.00	0.12	0.15	0.00	-												
[13]	0.09	0.00	0.01	0.00	0.00	0.03	0.03	0.00	0.12	0.14	0.01	0.00	-											
[14]	0.10	0.01	0.02	0.01	0.01	0.05	0.05	0.01	0.14	0.16	0.02	0.01	0.02	-										
[15]	0.10	0.09	0.11	0.09	0.09	0.11	0.11	0.09	0.11	0.13	0.10	0.09	0.09	0.11	-									
[16]	0.10	0.01	0.02	0.01	0.01	0.05	0.05	0.01	0.14	0.16	0.02	0.01	0.02	0.00	0.11	-								
[17]	0.09	0.00	0.01	0.00	0.00	0.03	0.03	0.00	0.12	0.15	0.00	0.00	0.00	0.01	0.09	0.01	-							
[18]	0.09	0.00	0.01	0.00	0.00	0.03	0.03	0.00	0.13	0.14	0.01	0.00	0.01	0.02	0.10	0.02	0.00	-						
[19]	0.10	0.01	0.02	0.01	0.01	0.05	0.05	0.01	0.14	0.16	0.02	0.01	0.02	0.00	0.11	0.00	0.01	0.02	-					
[20]	0.07	0.08	0.09	0.08	0.08	0.08	0.08	0.08	0.12	0.14	0.09	0.08	0.08	0.10	0.08	0.10	0.08	0.09	0.10	-				
[21]	0.10	0.09	0.11	0.09	0.09	0.11	0.11	0.09	0.13	0.15	0.10	0.09	0.10	0.08	0.07	0.08	0.09	0.10	0.08	0.07	-			
[22]	0.09	0.09	0.11	0.09	0.09	0.10	0.10	0.09	0.14	0.15	0.10	0.09	0.10	0.08	0.09	0.08	0.09	0.10	0.08	0.08	0.03	-		
[23]	0.08	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.13	0.15	0.10	0.10	0.10	0.08	0.11	0.08	0.10	0.10	0.08	0.09	0.06	0.05	-	
[24]	0.06	0.08	0.09	0.08	0.08	0.08	0.08	0.08	0.12	0.12	0.09	0.08	0.08	0.10	0.09	0.10	0.08	0.09	0.10	0.07	0.08	0.07	0.02	-

Note: [1] : *F. canicularis* (Beijing) GQ118368; [2] : *M. domestica* (Baotou) FJ763665; [3] : *M. domestica* (Changsha) GQ118387;
[4] : *M. domestica* (Chifeng) FJ763661; [5] : *M. domestica* (Datong) FJ763654; [6] : *M. domestica* (Fuzhou) GQ118376;
[7] : *M. domestica* (Fuzhou) GQ396683; [8] : *M. domestica* (Jianghua) FJ763691; [9] : *M. domestica* (Jishou) FJ763711;
[10] : *M. domestica* (Jishou) GQ396696; [11] : *M. domestica* (Linyi) FJ763695; [12] : *M. domestica* (Nanyang) FJ763679;
[13] : *M. domestica* (Shijiazhuang) FJ763657; [14] : *M. domestica* (Xian) GU145238; [15] : *M. domestica* (Xiangxiang) FJ763710; [16] : *M. domestica* (Yichun) GU145237; [17] : *M. domestica* (Yongzhou) FJ763685;
[18] : *M. domestica* (Yongzhou) GQ396693; [19] : *M. domestica* (Yongzhou) GU145239;
[20] : *M. hortorum* (Chifeng) GQ118375; [21] : *O. chalcogaster* (Hohhot) GU145258;
[22] : *O. chalcogaster* (Yongzhou) GU145260; [23] : *O. spinigera* (Changsha) GU145261;
[24] : *O. spinigera* (Nanning) FJ763660

were 14% and 8%, that of *O. spinigera* and *O. chalcogaster* were 8% and 5%, that of *O. spinigera* and *M. hortorum* were 9% and 7%, that of *O. chalcogaster* and *M. hortorum* 8% and 7%. Beside, the levels of divergence between *F. canicularis* and *M. hortorum* were 7%.

Table 3 show the mean value of interspecific variation between each species. The value between *M. domestica* and other species was from 9% to 10%, and the lowest value was between *O. spinigera* and *O. chalcogaster*, both of which were from the same genus *Ophyra*.

Intraspecific variation

The data matrix (Table 4) displays the maximum, minimum and mean intraspecific divergence of uncorrected percentage sequence. And the Table 2 show the detail of the intraspecific divergence of each species.

The mean intraspecific variation of *M. domestica* was 5%, and the maximum value was between the pairs of GQ118387 and GQ396696, GU145239 and GQ396696, GU145237 and GQ396696, GU145238 and GQ396696. In addition, the level of divergence between the pairs of GQ396696 and other specimens, FJ763711 and other specimens were higher, while the value between GQ396696 and FJ763711, which came from the same city Jishou, was low (2%). The intraspecific variation of *O. spinigera* and *O. chalcogaster* were 2% and 3% respectively.

Geographical variation

Besides calculating the level of the genetic variation, the variation of *M. domestica* based on the geographical reason was also analyzed in this study. The levels of divergence between every two different locations were showed in Table 5. The most apparent difference of the divergence values was between Jishou and other cities, and the values were from 9% to 11%. With regards to difference in latitude, the pairs of cities located at the same latitude: Datong (40.08°) and Baotou (40.66°), Yinchuan (38.46°) and Shijiazhuang (38.04°), Changsha (28.23°) and Jishou (28.30°), displayed the values of 0%, 1% and 11%.

Table 6 show the level of variability within the same city. Some species groups which included only one specimen were not mentioned in this table. The values between the same city were very low (≤1%), which indicated the similarity of the specimens from the same city.

DISCUSSIONS

Twenty-four specimens in this study were all collected from rat and pig carcasses placed on the outdoor grassland. And this study was conducted from July to September since 2006. Internationally, studies are usually carried out on pigs (Lopes *et al.*, 2001), rabbits (Bharti & Singh, 2003), dogs (Reed, 1958) and humans (Rodriguez & Bass, 1983). These studies indicated that the succession of insects on animals' carcasses was similar with that on human carcasses, and the difference only existed on the abundance of insects.

Table 3. Interspecific variation of specimens

	1	2	3	4	5
[1]					
[2]	0.10				
[3]	0.10	0.06			
[4]	0.10	0.10	0.07		
[5]	0.09	0.08	0.08	0.07	

Note: [1] *M. domestica*; [2] *O. chalcogaster*; [3] *O. spinigera*; [4] *F. canicularis*; [5] *M. hortorum*.

Table 4. Maximum, minimum and mean intraspecific variation expressed as a percentage of the total of 289base pairs of 16S rDNA data

species	Numbers of specimens	Max (%)	Min (%)	Mean (%)
M. domestica	18	16	0	5
O. spinigera	2	2	2	2
O. chalcogaster	2	3	3	3
M. hortorum	1	–	–	–
F. canicularis	1	–	–	–

Table 5. The geographical variation between every two locations

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
[1]														
[2]	0.00													
[3]	0.01	0.01												
[4]	0.01	0.01	0.00											
[5]	0.01	0.01	0.01	0.00										
[6]	0.01	0.01	0.00	0.00	0.00									
[7]	0.01	0.01	0.00	0.00	0.00	0.00								
[8]	0.01	0.01	0.00	0.00	0.00	0.00	0.00							
[9]	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00						
[10]	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.00	0.00					
[11]	0.08	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07				
[12]	0.11	0.11	0.10	0.10	0.09	0.10	0.10	0.10	0.10	0.10	0.09			
[13]	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.08	0.10		
[14]	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.08	0.11	0.03	

Note: [1] Yichun; [2] Xi_an; [3] Yongzhou; [4] Datong; [5] Shijiazhuang; [6] Chifeng; [7] Baotou; [8] Nanyang; [9] Jianghua; [10] Linyi; [11] Xiangxiang; [12] Jishou; [13] Fuzhou; [14] Changsha

Table 6. The geographical variation within locations

Location	Number of species	Max (%)	Min (%)	Mean (%)
Yongzhou	3	1	0	1
Jishou	2	1	1	1
Fuzhou	2	0	0	0

The standard of decomposition used in this study was defined by Tullis & Goff (1987). During the study, the insects of Muscidae family were observed throughout the process of decomposition, except the dry stage of decomposition. Adults of *M. domestica* often visited the carcasses at an early stage of decomposition, such as bloat and active stages. These results were in agreement with Sharanowski *et al.* (2008). And the absence of larvae of *M. domestica* was also reported by 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). The numbers of *M. hortorum* and *F. canicularis* were relatively small, and they were all observed at bloat stage of decomposion.

In this study, the DNA extraction and sequencing of all specimens were finished. And the species of Muscidae family could be distinguished through the variation in 16S rDNA sequence. We assessed the 16S rDNA sequence as a potential marker for the identification of Muscidae family flies from China. And the results indicated that the used technique is as effective as morphological method in identification of Muscidae species, while, in order to acquire correct identification, the morphologic method needs expertise in specialized taxonomy (Leclercq & Lecomte, 1978), yet the technology using mtDNA is easier to perform and saves time. Moreover, the limited amount of the insect tissue in this study made the possibility for further morphological study and genetic analyses.

The monophyletic separation of *M. domestica* in the phylogenetic tree (Figure 2) confirmed the sufficient resolution of the genetic marker. Some specimens from the same city were grouped together with high bootstrap values, such as those from Fuzhou and Jishou, while the specimens from Yongzhou were not in one clade. Also the species of *O. spinigera* and *O. chalcogaster* which formed two single clade respectively with high support value of 52% indicated the similarity of these two species. *Morellia hortorum* and *F. canicularis* were in the same clade, and

the low support value of 40% enabled to distinguish these two species.

In the interspecific variation analysis, the lowest value was between *O. spinigera* and *O. chalcogaster* from the same genus *Ophyra*, and the values between other species were higher, which indicated the efficacy of 16S rRNA to idenify the species from different genera of Muscidae family. Furthermore, the value between *O. spinigera* and *O. chalcogaster* of the same genus *Ophyra* was larger than 5%, which could distinguish these two species. This also supported the finding of Sun *et al*. (2006) who used the 551 bp region of the gene of 16S rRNA to effectively identify *Lucilia sericata* and *Lucilia cuprina* from the same genus *Lucilia*.

Table 2 and Table 4 show the intraspecific variation analysis. The value of *M. domestica* varied in a wide range, from 0% to 16%. The highest values were from the pairs of specimens collected from Jishou and other locations, from 11% to 16%. And the variation value between the two specimens from Jishou was low (2%). This might be because the mountainous circumstance of Jishou prevents the migration of the species. Besides, the specimen from Xiangxiang displayed a high variation value with others, and more samples are needed from this location to explain this disparity. The mean level of the intraspecific was 5%, which showed the difference within the *M. domestica* species.

The maximum mean intraspecific variability for all specimens was 5%, while the minimum interspecific variability was 6%, this difference between the threshold levels enabled differentiation to be observed between forensically important Muscidae species in China.

Geographical variation is one of the evidence to deduce the geographical origin of important forensic insects species. In addition, it can also determine the scene of crime (SOC). Table 5 shows the geographical variation of *M. domestica*. The results showed that the variation values differed as latitude changed. The value of the location pairs at the same latitude was low. However, the specimens' pairs collected from Jishou and Changsha displayed a high value (11%). This might be because Jishou is a mountainous city; while Changsha is a plain for a wider range of migration, which was proven by the relatively low value between Changsha and other plain-locations of the similar latitude. The values between Fuzhou and other locations were a little higher ($\geq$2%), which could be explained by the inshore position of the city. Furthermore, in Table 6, the low value of the pairs in the same cities showed the conservation of *M. domestica* within one location. In the study of Marquez & Krafsur (2002), they also noted that differentiation of *M. domestica* between regions was greater than differentiation within regions.

The 289 bp fragment of the mitochondrial 16S rRNA gene in this study displayed that, besides the morphological method of identification, this region has potential as a discriminatory tool in identification of Muscidae family flies. Future work with more Muscidae family species from other parts of China could indicate the examination of more variable mitochondrial genes, and then improve the molecular method for identification of forensically important Muscidae species.

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REFERENCES

Arnaldos, M.I., García, M.D., Romera, E., Presa, J.J. & Luna, A. (2005). Estimation of postmortem interval in real cases based on experimentally obtained entomological evidence. *Forensic Science International* **49:** 57–65.

An, R.S., Tan, S.J. & Chen, X.F. (2003). Improvement in grinding tissue during extracting DNA from small insects. *Entomological Knowledge* **39:** 311–312.

Benecke, M. & Wells, J.D. (2001). DNA techniques for forensic entomology. In: *Forensic Entomology: Utility of Arthropods in Legal Investigations.* Byrd. Boca Raton: CRC Press, pp.341–352.

Bharti, M. & Singh, D. (2003). Insect faunal succession on decaying rabbit carcasses in Punjab, India. *Journal of Forensic Sciences* **48:** 1133–1143.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783–791.

Greenberg, B. (1973). Flies and disease. In: Biology and disease transmission. Princeton: Princeton University Press, pp.447.

Heo, C.C., Mohamad, A.M., Jeffery, J., Kurahashi, H. & Omar, B. (2008). On the occurrence of *Musca domestica* L. oviposition activity on pig carcass in peninsular Malaysia. *Tropical Biomedicine* **25:** 252–253.

Keh, B. (1985). Scope and applications of forensic entomology. *Annual Review of Entomology* **30:** 137–154.

Leclercq, M. & Lecomte, J. (1978). Emergency treatment of insect bite complications. *Revue Médicale de Liège* **33:** 240–249.

Linhares, A.X. (1981). Synanthropy of Muscidae, Fanniidae and Anthomyiidae (Diptera) in the city of Campinas, São Paulo, Brazil. *Revista Brasileira de Entomologia* **25:** 231–243.

Lopes, de., Carvalho, L.M. & Linhares, A.X. (2001). Seasonality of insect succession and pig carcass decomposition in a natural forest area in southeastern Brazil. *Journal of Forensic Sciences* **46:** 604–608.

Marquez, J.G. & Krafsur, E.S. (2002). Gene flow among geographically diverse housefly populations (*Musca domestica* L.): a worldwide survey of mitochondrial diversity. *The Journal of Heredity* **93:** 254–259.

Matuszewski, S., Bajerlein, D., Konwerski, S. & Szpila, K. (2008). An initial study of insect succession and carrion decomposition in various forest habitats of Central Europe. *Forensic Science International* **180:** 61–69.

Nurita, A.T., Abu Hassan, A. & Nur Aida, H. (2008). Species composition surveys of synanthropic fly populations in northern peninsular Malaysia. *Tropical Biomedicine* **25:** 145–153.

Omar, B., Mohd, A.M., Sulaiman, S. & Oothuman, P. (1994). Dipteran succession in monkey carrion at a rubber tree plantation in Malaysia. *Tropical Biomedicine* **11:** 77–82.

Reed, H.B. (1958). A study of dog carcass communities in Tennessee with special reference to the insects. *The American Midland Naturalist Journal* **59:** 213–245.

Rodriguez, W.C. & Bass, W.M. (1983). Insect activity and its relationship to decay rates of human cadavers in east Tennessee. *Journal of Forensic Sciences* **28:** 423–432.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406–425.

Sharanowski, B.J., Walker, E.G. & Anderson, G.S. (2008). Insect succession and decomposition patterns on shaded and sunlit carrion in Saskatchewan in three different seasons. *Forensic Science International* **179:** 219–240.

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.

Smith, K.G.V. (1986). A manual of forensic entomology. In: British Museum (Natural History). Ithaca: Cornell University Press. pp.205.

Sulaiman, S., Sohaidi, A.R., Hashim, Y. & Iberahim, R. (1988). The role of some cyclorrhaphan flies as carriers of human helminthes in Malaysia. *Medical Veterinary Entomology* **2:** 1–6.

Sun, X.M., Cai, J.F., Ying, B.W., Fang, Y.Q., Yun, L.B., Yuan, W.A., Gao, S. & Hou, Y.P. (2006). Identification of Sarcosaphagous Flies by Analyses the Sequence of 16S rDNA, *Journal of Forensic Medicine* **22:** 36–38.

Tabor, K.L., Fell, R.D. & Brewster, C.C. (2005). Insect fauna visiting carrion in Southwest Virginia. *Forensic Science International* **150:** 73–80.

Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10:** 512–526.

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 of the United States of America* **101:** 11030–11035.

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.

Tullis, K. & Goff, M.L. (1987). Arthropod succession in exposed carrion in a tropical rainforest on O'ahu Island, Hawai'i. *Journal of Medical Entomology* **24:** 332–339.

Wang, J., Li, Z., Chen, Y., Chen, Q. & Yin, X. (2008). The succession and development of insects on pig carcasses and their significances in estimating PMI in south China. *Forensic Science International* **179:** 11–18.

Wells, J.D. & Williams, D.W. (2007). Validation of a DNA-based method for identifying Chrysomyinae (Diptera: Calliphoridae) used in a death investigation. *International Journal of Legal Medicine* **121:** 1–8.

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. *American Journal of Forensic Medicine and Pathology* **28:** 308–313.