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The availability of 16SrDNA gene for identifying forensically important blowflies in China

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Abstract: Different species of sarcosaphagous insects are found on human corpses at different phases after death, which is important for the estimation of postmortem interval (PMI) and other questions of forensic relevance. Calliphoridae are one of the earliest visitors to infest a corpse and lay their eggs, and develop maggots. Following the conventional method by morphology, a molecular method is a necessary assistance of species identification. This study has investigated the applicability of sequencing of mitochondrial DNA (mtDNA) based on a 250 base pair region of the gene for 16S rDNA to identify the forensically important species of Calliphoridae which were collected from 10 sites distributed at 9 provinces in China, including 25 specimens (8 species) in 3 genera of Calliphoridae family, including some specimens of *Chrysomya megacephala* (Fabricius), *Chrysomya rufifacies* (Macquart), *Calliphora vicina* (Robineau-Desvoidy), *Lucilia caesar* (Linnaeus), *Lucilia porphyrina* (Walker), *Lucilia sericate* (Meigen), *Lucilia bazini* (Seguy), *Lucilia illustris* (Meigen). The analysis of *16S rDNA* sequences revealed abundant phylogenetically informative nucleotide substitutions that could identify Calliphoridae to species group. It renders the reliable identification of important sarcosaphagous insects in China, particularly in case of murder or suspicious death by providing an estimate of post-mortem interval (PMI) and scene of crime.

Key words: Forensic entomology; Calliphoridae; 16S ribosomal DNA; Species identification; China

Forensic entomology—the science of study insects and arthropods related to legal issues is important evidence connected with death investigation in court trials [1]. The use of different species of insects found on the human corpses at different phases after death as forensic indicators is increasingly applied around the world. The entomological evidence has been played a valuable role in the investigation of murders and suspicious deaths and helping to answer questions relating to estimation of the postmortem interval (PMI), postmortem transfer, diagnosis of poisoning (in case of total absence of soft tissue) and neglect of living people [2] and further to determine time, manner and place of death [3] for the police service and the justice system [4].

Calliphoridae adults are usually the earliest one of sarcosaphagous insects to come in contact with a corpse [5]. So the first step is to identify the species of Calliphoridae accurate, which is conventionally done by morphological features. The morphological similarity poses a great challenge

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for forensic entomologists, and up to now, it is still difficult to diagnostic certain groups within Calliphora at their egg, pupa and larval stages by using morphological criteria [6]. The immature may require rearing to adult stage for accurate identification, which is a time-consuming process and the form of the evidence would change [7]. Under these circumstances, species identification based on genetic examination is a better option.

Compared with nuclear DNA, mtDNA has relatively higher level of variation in this region [8], maternal inheritance, rapid rate of divergence and lack of recombination [9]and can be isolated more easily [11]. So it became a better option for species discrimination. In the past years, the study of the mtDNA were focused on Cytochrome oxidase I gene (*COI*) and Cytochrome oxidaseII gene (*COII*) [7, 8, 10, 12-18]. For this study, portions of the 16S rDNA mitochondrial genes were selected for amplication, and the analyses of amplication, 16S rDNA prensent an available method of differentiation and classification of the insects. With the same as *COI* and *COII*, *16S rDNA* is a molecular marker existing in mitochondria and has a high conserved region. Unlike *COI* and *COII*, *16S rDNA* was widely used to analyse the relationship of edge species and the higher categories of different insects[19].

In recent studies, Liu *et al* analysed the phylogenetic relationships among taxa within Acrididae by using the combined sequence data of *16S rDNA*, which revealed abundant phylogenetically informative nucleotide substitutions that could identify insects into species group[20]. Sun Xiao-ming and Cai Ji-feng has successfully identified the *Lucilia sericata* (Meigen) and *Lucilia cuprina* (Wiedemann) by 551 bp of regions of the gene encoding for old *16S rDNA*, which was likely to be a successful compliment to identify the sarcosaphagous flies by sequence analysis of *COI* and *COII* in mtDNA [21]. In this study, we determined to develop a database of *16S rDNA* sequence data which makes it possible to identify important species of family Calliphoridae in China. By using a new primer of *16S rDNA*, we can amplify a sequence of only 250 bp which is much shorter than the one used before. The improved primer of *16S rDNA* can be used to reassess the phylogenetic relationship about 10 species of family Calliphoridae. And the primer is also available for clarifying unresolved issues such like this.

2. Material and methods

2.1 Specimens

Specimens of blowflies were collected from 10 sites distributed at 9 provinces in China, and all samples were trapped using animal (rabbit, dog or pig) corpse-baited traps either by the authors or by students of the Central South University and stored at room temperature. They were identified using traditional morphological characters by using an identification key to adult Calliphoridae[22]. Specimens used in this study were listed in Table 1.

2.2. DNA extraction

The adult flies of thorax muscles were removed and subjected to mtDNA extraction by using the improvement in grinding tissue during extracting DNA from small insects [23]. 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, and the head and abdomen of each specimen was retained to recheck its identity and further molecular analysis.

2.3 PCR conditions and purification of PCR products

A portion of 250 bp of the *16S rDNA* gene was amplified and sequenced by using forward primers 5'-CGCTGTTATCCCTAAGGTAA-3' and reverse primers 5'-CTGGTATGAAAGG TTTGACG-3' primers, which were designated by us. The primers were designed by software primer 5 which based on homologous analysis of published relevant mtDNA sequences of some different insect species.

The PCR reaction volume for each locus was 25μl, containing 1-5μl (20-40ng) of template DNA, 12.5μl 2×GoTaq® Green Master Mix (containing 4μl dNTP (1mmol/ml), 1.0u Taq polymerase, 2.5μl 10×buffer (Mg2+1.5mmol/l), produced by Promerga, Madison, WI, USA), 0.25-2.5μl each primer (10μM), Nuclease-Free Water added to a total volume of 25 μl. PCR amplifications were performed in a thermocycler (Perkin-Elmer9600), with initiative denaturing for 3 minutes at 94℃, followed by 38 cycles of 94℃ for 30 seconds, 50.8℃ for 30 seconds and 72℃ for 30 seconds.

2.4. Sequencing

After purification of the PCR products with QIA-quick columns cycle, sequencing was performed on both forward and reverse strands through using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Removal of excess dye-deoxyterminators primers and buffer was accomplished by DYE-EX spincolumns (Qiagen).

2.5 Phylogenetic analysis

Phylogenetic systematic analysis is the study of the relationships between different groups of organisms, and is thus based on similarity and difference in the chosen characters (see [24]for further phylogenetic definition). As the sequences were conservation and did not contain any insertions or deletions, all resultant sequences in this study were aligned using ClustalW (http://www.ddbj.nig.ac.Jp/E-mail/clastalw-e.html). And the obtained sequences have been deposited in GenBank by Sequin (http://www.ncbi.nlm.nih.Gov/equin/index.html).

We tested whether the sequences were of mitochondrial origin or represented paralogous sequences resident in the nucleus [25] in two ways through using MEGA4 [26]. Sequences were translated with the sarcosaphagous flies mitochondrial genetic code and, since nuclear paralogues can lose their coding function, the predicted amino acid sequences were inspected for inappropriate stop codons. We also compared the base composition of the individual sequences since nuclear paralogues can have divergent base compositions relative to mitochondrial genes.

The multiple-alignment program clustal X was used to carried out the sequence alignments. A neighbor-joining tree using the Tamura and Nei [27] model of nucleotide substitution was constructed through using the MEGA package [28]. Bootstrap values were calculated by repeated random sampling of the data to provide an indication of the confidence limited for a particular grouping.

3. Results

A total of 25 individuals were sequenced and aligned 250 bp of the *16S rDNA* gene. The estimated sequences are deposited in gene bank (http://www.ncbi.nlm.nih.gov) under the accession numbers in table 1.

Table 1. Information on specimens used in this study

Species	Accession number	Locality and coordinates	No
Group1 Lucilia			
Lucilia sericata (Meigen)	GU145217	Urumuqi, Xingjiang (43°45′N 87°37′E)	1
	GU145229	Urumuqi, Xingjiang (43°45′N 87°37′E)	2
	GU145226	Xining, Qinghai (36°34′N 101°49′E)	3
	GU145230	Xining, Qinghai (36°34′N 101°49′E)	4
	GU145218	Xining, Qinghai (36°34′N 101°49′E)	5
	GU145219	Xian, Shanxi (34°00′N 108°04′E)	6
	GU145222	Weifang, Shandong (36°41′N 119°10′E)	7
	GU145236	Yongzhou, Hunan (24°40′N 111°25′E)	8
	GU145228	Hohhot, Inner mongaolia (40°48′N 111°41′E)	9
Lucilia bazini (Seguy)	GU145224	Zhangjiajie, Hunan (38°04′N 114°51′E)	10
Lucilia illustris (Meigen)	GU145231	Xian, Shanxi (19°32′N 110°20′E)	11
Lucilia porphyrina (Walker)	GU145225	Changsha, Hunan (28°12′N 112°59′E)	12
Lucilia caesar (Linnaeus)	GU145220	Hohhot, Inner mongaolia (40°48′N 111°41′E)	13
	GU145227	Xian, Shanxi (34°00′N 108°04′E)	14
Group2 Calliphora			
Calliphora vicina (Robineau-Desvoidy)	GU145240	Urumuqi, Xingjiang (43°45′N 87°37′E)	15
	GU145242	Wuhu, Anhui (31°20′N 119°21′E)	16
	GU145241	Xining, Qinghai (36°34′N 101°49′E)	17
Group3 Chrysomya			
Chrysomya rufifacies (Macquart)	GU145223	Yongzhou, Hunan (24°40′N 111°25′E)	18
	GU145263	Weifang, Shandong (36°41′N 119°10′E)	19
Chrysomya megacephala (Fabricius)	GU145256	Zhangjiajie, Hunan (28°60′N 110°40′E)	20
	GU145262	Yichun, Jiangxi (27°47′N 114°38′E)	21
	GU145252	Yichun, Jiangxi (27°47′N 114°38′E)	22
	GU145253	Hohhot, Inner Mongaolia (40°48′N 111°41′E)	23
	GU145254	Shijiazhuang, Hebei (38°04′N 114°51′E)	24
	GU145255	Yongzhou, Hunan (24°40′N 111°25′E)	25

* All samples were gathered from 10 sites distributed at 9 provinces in China, including 25 specimens (8 species) (1-9 *Lucilia sericata*; 10 *Lucilia bazini*; 11 *Lucilia illustris*; 12 *Lucilia porphyrina*; 13, 14 *Lucilia caesar*; 15-17 *Calliphora vicina*; 18, 19 *Chrysomya rufifacies*; 20-25 *Chrysomya megacephala*) of insects respectively.

3.1. Morphological identification of specimens for 16S rDNA analysis

The 25 samples were belonging to the Calliphoridae family. All of the samples were collected from 10 different places of 9 provinces in China in last 3 years. Before the experiment, we had identified all the specimens morphologically according to the traditional morphological classification.

As one of the most important sarcosaphagous insects, blowflies often reach the corpse in first. All the specimens we collected and choose for this study belong to the Calliphoridae family are divided into 3 genus and 8 species: (1) Chrysomya: *Chrysomya megacephala* (6 specimens), *Chrysomya ruffifacies* (2 specimens); (2) Calliphora: *Calliphora vicina* (3 specimens); (3) Lucilia: *Lucilia caesar* (2 specimens), *Lucilia porphyrina* (1 specimens), *Lucilia sericate* (9 specimens).

The altogether 25 specimens were sequenced over *16S rDNA* regions. The morphological identification and original locality of all the 25 specimens were showed in the table 1.

3.2. 16S rDNA phylogenetic analysis

The neighbor-joining (NJ) tree was based on the *16S rDNA* sequence data analyzed with Kimura's two-parameter model. The optimal tree with the sum of branch length (5.66527772) was shown (Fig 1).

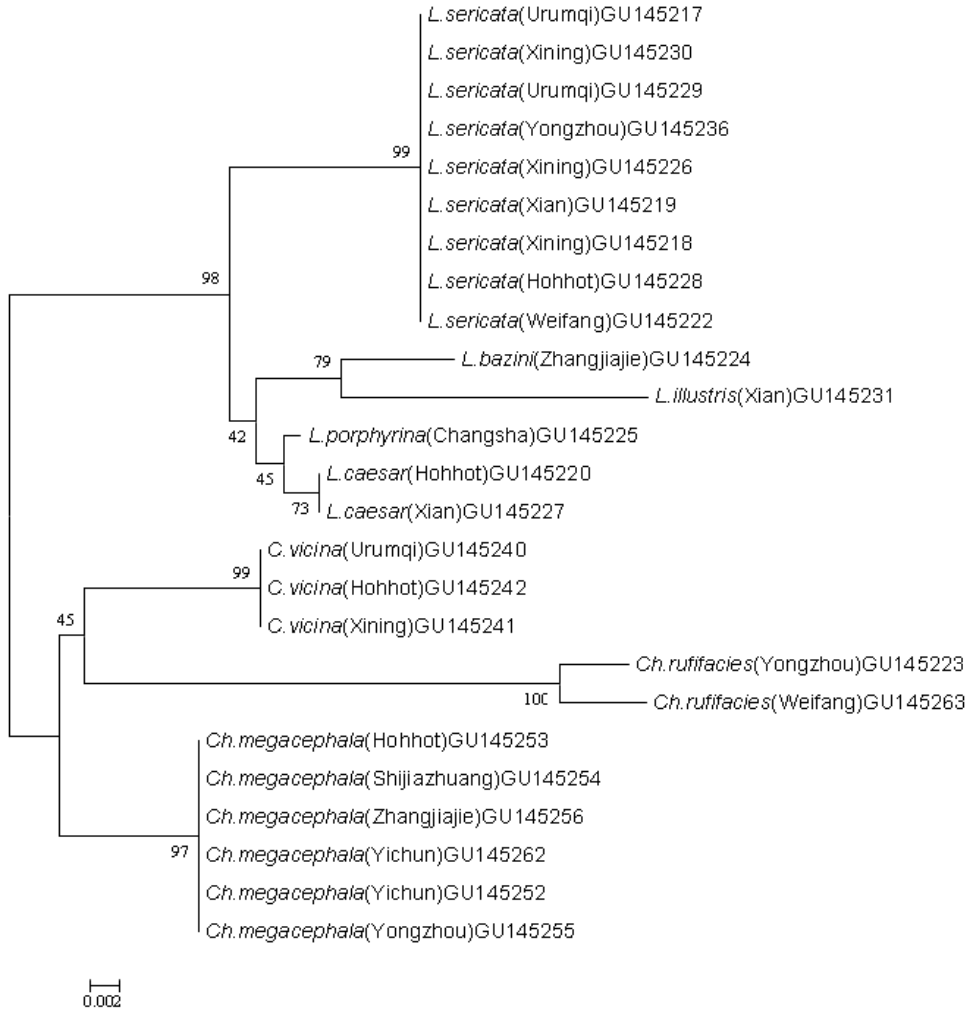

Figure 1. NJ tree displayed evolutionary relationships of 25 taxa. Bootstrap values indicate support for nodes. The bar indicates 0.005 substitutions per site. (Chrysomya megacephala, Chrysomya rufifacies, Calliphora vicina, Lucilia caesar, Lucilia porphyrina, Lucilia sericata, Lucilia basini, Lucilia illustris. abbreviated as Ch. megacephala, Ch. rufifacies, C. vicina, L. caesar, L. porphyrina, L. sericata, L. basini, L. illustris)

A total of 250 aligned sites for the 25 *16S rDNA* sequences were included in the following. The consensus tree was computed, and the bootstrapped version resulted in the same tree as gained using neighbor-joining.

According to the neighbor-joining (NJ) tree based on primer *16S rDNA*, all the specimens were classified into four main groups with high bootstrap support: all the *Chrysomya megacephala* specimens were grouped together with 97% support; *Chrysomya ruffifacies* were grouped together with full bootstrap support; *Calliphora vicina* formed a single cluster with 99% support, five species of the genus Lucilia genus (contains *Lucilia caesar*, *Lucilia bazini*, *Lucilia illustris*, *Lucilia porphyrina*, *Lucilia sericate*) were grouped together with a value of 98%. Within the Lucilia genus clade, the specimens of *Lucilia sericate* were clustered together with 99% support. *Lucilia caesar* formed a single group. Species of *Lucilia porphyrina* and *Lucilia caesar* formed a separate group but at a low bootstrap.

3.3. Intraspecific variation

A total of 250 aligned sites for the 25 *16S rDNA* sequences were included in the phylogenetic analyses by Kimura 2-parameter distance method. Table 2 showed the divergence value between every two specimens within Calliphoridae family. Uncorrected percentage sequence divergence among all taxa ranged from 0 to 8.25%. Table 3 showed the level of *16S rDNA* gene nucleotide divergence within a group. *Chrysomya rufifacies* had the maximum intraspecific variation, which was 1%, the other intraspecific variation of other species were 0%.

Table 2. Pirwise distances for the analyzed regions of *16SrDNA*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
[1]																									
[2]	0.00																								
[3]	0.00	0.00																							
[4]	0.00	0.00	0.00																						
[5]	0.00	0.00	0.00	0.00																					
[6]	0.00	0.00	0.00	0.00	0.00																				
[7]	0.00	0.00	0.00	0.00	0.00	0.00																			
[8]	0.00	0.00	0.00	0.00	0.00	0.00	0.00																		
[9]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00																	
[10]	2.47	2.47	2.47	2.47	2.47	2.47	2.47	2.47	2.47																
[11]	3.92	3.92	3.92	3.92	3.92	3.92	3.92	3.92	3.92	2.82															
[12]	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.39	2.82														
[13]	2.12	2.12	2.12	2.12	2.12	2.12	2.12	2.12	2.12	1.75	3.19	0.35													
[14]	2.12	2.12	2.12	2.12	2.12	2.12	2.12	2.12	2.12	1.75	3.19	0.35	0.00												
[15]	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.35	6.62	3.61	3.23	3.23											
[16]	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.35	6.62	3.61	3.23	3.23	0.00										
[17]	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.38	4.35	6.62	3.61	3.23	3.23	0.00	0.00									
[18]	7.11	7.11	7.11	7.11	7.11	7.11	7.11	7.11	7.11	7.83	8.25	6.29	6.26	6.26	4.71	4.71	4.71								
[19]	7.08	7.08	7.08	7.08	7.08	7.08	7.08	7.08	7.08	7.79	8.21	6.26	6.23	6.23	5.06	5.06	5.06	1.05							
[20]	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	4.70	5.46	3.23	3.22	3.22	2.47	2.47	2.47	4.35	4.70						
[21]	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	4.70	5.46	3.23	3.22	3.22	2.47	2.47	2.47	4.35	4.70	0.00					
[22]	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	4.70	5.46	3.23	3.22	3.22	2.47	2.47	2.47	4.35	4.70	0.00	0.00				
[23]	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	4.70	5.46	3.23	3.22	3.22	2.47	2.47	2.47	4.35	4.70	0.00	0.00	0.00			
[24]	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	4.70	5.46	3.23	3.22	3.22	2.47	2.47	2.47	4.35	4.70	0.00	0.00	0.00	0.00		
[25]	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	3.99	4.70	5.46	3.23	3.22	3.22	2.47	2.47	2.47	4.35	4.70	0.00	0.00	0.00	0.00	0.00	

†The number in this table is corresponding with table1.

3.4. Interspecific variation

Pairwise divergence between species was calculated and showed in Table 4. Variation was calculated based on an average computed from all individuals of the species. The maximum and minimum level of divergence between every two species was 0.35% and 8.23%. The maximum level of interspecific variation was found in species *Lucilia illustris* and *Chrysomya rufifacies*. Species pairs in Lucilia genus were separated at a low variation, such as *Lucilia porphyrina*/*Lucilia sericata*, *Lucilia porphyrina*/*Lucilia bazini*, *Lucilia caesar*/*Lucilia bazini* displayed interspecific variation as 1.76%, 1.39% and 1.75% respectively. *Lucilia caesar*/*Lucilia illustris* displayed 3.19% divergence. However sister species *Lucilia caesar* and *Lucilia porphyrina* was differed by only 0.35%.

Table 3. Intraspecific variation for each species

Species	Number of specimens	Mean (%)
Lucilia sericata	9	0.00
Lucilia caesar	2	0.00
Calliphora vicina	3	0.00
Chrysomya rufifacies	2	1.00
Chrysomya megacephala	6	0.00

Table 4 Pairwise divergence between different species expressed within Calliphoridae family

Species	1	2	3	4	5	6	7
1 *Lucilia sericata*							
2 *Lucilia bazini*	2.47						
3 *Lucilia illustris*	3.92	2.82					
4 *Lucilia porphyrina*	1.76	1.39	2.82				
5 *Lucilia caesar*	2.12	1.75	3.19	0.35			
6 *Calliphora vicina*	4.38	4.35	6.62	3.61	3.23		
7 *Chrysomya rufifacies*	7.10	7.81	8.23	6.28	6.25	4.88	
8 Chrysomya megacephala	3.99	4.70	5.46	3.23	3.22	2.47	4.52

‡Note: 1. *Lucilia sericata*, 2. *Lucilia bazini*, 3. *Lucilia illustris*, 4. *Lucilia porphyrina*, 5. *Lucilia caesar* were under *Lucilia genus*; 6. *Calliphora vicina* were under Calliphora genus; 7. *Chrysomya rufifacies*, 8. *Chrysomya megacephala* under Chrysomya genus.

4. Discussions

mtDNA is recognized as a useful tool for evolutionary study because of its relatively higher mutation rate than nuclear DNA, and also of the presence of both conserved and variable segments [27]. When morphology is compromised, genetic species identification attempts to match an unknown evidence sample to a known reference sample by comparing sequences of genes, usually mitochondrial DNA (mtDNA) loci that are known to vary between species [29]. The forensically important Calliphoridae were the first sarcosaphagous insects to be distinguished by using DNA-based identification techniques in 1994 [10].

In this study, through the sequence measurement and pairwise analysis of the 250 bp region of *16S rDNA*, we can successfully distinguish the Calliphoridae species collected from different locations of China. The results of mtDNA sequence analysis corresponded with the morphologic method. The results improved the method of using the sequences of every species obtained from the study to the application of case investigation. And it was effective evidence that is not only reducing the difficulty of identification of adult blow flies but also saving the time of solving cases.

The specimens from Changsha were fresh, while other specimens (during 2007-2009) were at different developmental stages (adults, larvae and pupae) and were stored in air-dried condition. All the 25 specimens were successfully extracted and sequenced. All the 250 aligned sites for the 25 *16S rDNA* sequences were analyzed to confirm that the new DNA-based methods can be applied on blowflies from China.

Figure 1 showed the NJ tree constructed from *16S rDNA* sequence data. Phylogenetic analysis was performed to examine the ability of the region to resolve species' identification and taxonomic relationships between species of Calliphoridae family. The support value for each cluster was provided by bootstrap values through random resampling data. In the species level, all the specimens in the same species were assigned to their respective species with high bootstrap supports, which illustrates the potential of *16S rDNA* sequence for use in interspecific distinction.

The five species of the genus Lucilia in this study were grouped with high bootstrap support. Speciemens of Lucilia sericate formed a single cluster with 99%. Other specimens were also well separated except *Lucilia caesar*/*Lucilia porphyrina* in the figure 1. *Lucilia caesar* and *Lucilia illustris* could not be distinguished by using *COI* in the past studies [30-32], however, according to this study; the two species were well separated by 3.2% interspecific variation. This is a compliment to the species identification by molecular methods. However, the species pairs *Lucilia caesar*/*Lucilia porphyrina* could not be distinguished satisfactorily by comparing the chosen *16S rDNA* sequence (Supplementary Table 4), though the two species were grouped together in NJ-tree. The deviations from reciprocal monophyly we observed can not be excluded from misidentified specimen.

Table 3, 4 and 5 showed the intraspecific variation and interspecific variation between species, and the pairwise calculation were corresponded to the NJ tree. The analyses by neighbor-joining methods showed the potential of this region to provide the necessary species-level identifications. The average value of the variation within the same species was from 0%-1%, and the average value of the variation between different species was from 1.39% to 8.23% except *Lucilia caesar*/*Lucilia porphyrina*, which implied the variation within the same species were lower than the variation of different species. This

supports the finding of Wells & Sperling [33] of intraspecific divergence of ≤1%, but not support the interspecific divergence of ≥3% by *COI*. Our study indicates that the ranges of variation of *16S rDNA* sequence data are a little different from that of *COI*. So a distinct range available for the variation of intra- and interspecific levels should be resolved in future study.

Chrysomya rufifacies is primarily a tropical fly, which is one of the most significants in the field of forensic entomology and the geographical structuring of the *Chrysomya rufifacies* is linked with unusually high intraspecific variation because of the variable behaviour result of ecological interaction with other carrion-colonizing species [34]. But according to our study, the intraspecific variation within *Chrysomya rufifacies* was 1% and the bootstrap support of specimens within this group was 100%. This is improved species *Chrysomya rufifacies* can be well distinguished by using the 250 aligned sites of *16S rDNA* sequences.

Analyzing geographical variation is an evidence to deduce the region divergence of important forensic insects' species and further to determine the scene of crime (SOC). Though some studies reported the differentiation of samples from different fields, according to Stijn Desmyter and Matthias Gosselin [35], 25 Chrysomyinae specimens, which were collected from different sites of France and analyzed based on the *COI* region, showed a very similar mitotypes with the Belgian ones.

Meanwhile the specimens from outside of Europe revealed no intraspecific geographical variation within *Chrysomya albiceps* and *Protophormia terraenovae*. Cai et al [36] concluded the correlation between the genetic distance and geographical difference of *Lucilia sericata* based on the 635 bp fragment of COII region. The clade of *Chrysomya megacephala* and *Lucilia sericata* in the study were also deprecated with well supported, but the two species in this study displayed no obvious geographically differentiation because the samples is limited.

As *Chrysomya megacephala* and *Lucilia sericata* are both the most common sarcosaphagous flies in China, more specimens and observations are needed to discuss the geographical variation of forensically important insects.

The results indicate that the morphological method is as effective as mtDNA sequences method in species identification of adult Calliphoridae species. But the specimens of *Lucilia caesar* and *Lucilia porphyrina* were still difficult to distinguish. In most situations, the species that are difficult or impossible to distinguish based on anatomical characters are close relatives. However, the morphological method requires expertise in specialized taxonomy to assure correct identification. Whereas the technology of molecular identification method by mtDNA has come to maturity, cost effective, flexible and easily accessible even in ordinary laboratories.

5. Conclusion

According to this study, the 250 base pair region of the *16S rDNA* displayed the potential of mtDNA for using as a discriminatory tool in forensic investigations and it was used to give an addition to the conventional methods. All the species of the Calliphoridae family were separated clearly in the NJ tree. The interspecific variation within the same genus was lower than specimens of other genus from the result of pairwise distance calculation. All one empty spare too much intraspecific variation is ≤1%, and the interspecific variation is ≥1.4%. According to this, the new region of the *16S rDNA* has the same effect as the 513 base pair region of the *16S rDNA*.

Although the results of this study demonstrate the value of the *16S rDNA* in general, the closely related species pairs such as *Lucilia caesar* and *Lucilia porphyrina*, *Calliphora vomitoria* and *Calliphora vicina* displayed a low level of interspecific variation, a larger region of the *16S rDNA* need to be sequenced for the classification in further study.

6. Acknowledgments

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