Insect science

Insect Science (2011) 18, 554–564, DOI 10.1111/j.1744-7917.2010.01377.x

ORIGINAL ARTICLE

Identification of forensically important blow fly species (Diptera: Calliphoridae) in China by mitochondrial cytochrome oxidase I gene differentiation

Qin-Lai Liu[1], Ji-Feng Cai[1,2], Yun-Feng Chang[1], Yan Gu[1], Ya-Dong Guo[1], Xing-Hua Wang[1], Ji-Fang Weng[1], Ming Zhong[1], Xiang Wang[1], Li Yang[1], Kun-Lu Wu[1], Ling-Mei Lan[1], Jiang-Feng Wang[4] and Yao-Qing Chen[1,3]

[1]*School of Basic Medical Sciences, Central South University,* [2]*Second Xiang-Ya Hospital of Central South University,* [3]*The Changsha Public Security Bureau in Hunan, Changsha, Hunan and* [4]*Department of Forensic Science and Technology, Guangdong Police College, Guangdong, China*

Abstract Unambiguous and rapid sarcosaphagous insect species identification is an essential requirement for forensic investigations. Although some insect species are difficult to classify morphologically, they can be effectively identified using molecular methods based on similarity with abundant authenticated reference DNA sequences in local databases. However, local databases are still relatively incomplete in China because of the large land area with distinct regional conditions. In this study, 75 forensically important blow flies were collected from 23 locations in 16 Chinese provinces, and a 278-bp segment of the cytochrome oxidase subunit I gene of all specimens was successfully sequenced. Phylogenetic analysis of the sequenced segments showed that all Calliphorid specimens were properly assigned into nine species with relatively strong supporting values, thus indicating that the 278-bp cytochrome oxidase subunit one region is suitable for identification of Calliphorid species. The clear difference between intraspecific threshold and interspecific divergence confirmed the potential of this region for Calliphorid species identification, especially for distinguishing between morphologically similar species. Intraspecific geographic variations were observed in *Lucilia sericata* (Meigen, 1826) and *Lucilia caesar* (Linnaeus, 1758).

Key words Calliphoridae, China, cytochrome oxidase subunit I (COI), forensic entomology, species identification

Introduction

Sarcosaphagous insect species infest and colonize human and animal remains in a predictable sequence over time (Amendt *et al.*, 2004; Anderson, 2004). The precise species identification of every insect sample collected from criminal scenes play an essential role in the accurate estimation of post mortem interval (PMI), especially when information on the post mortem phenomena is not available. Traditional morphological keys are unavailable or difficult to use for many immature stages of these insects and even for adult specimens (Stevens & Wall, 2001; Wallman & Donnellan, 2001; Saigusa *et al.*, 2009). Morphologic methods may yield definitive identification by rearing larvae to adults, but this approach can be time-consuming and requires larvae to be collected live and kept in conditions suitable for continued development (Ames *et al.*, 2006). Molecular methods can

Correspondence: Ji-Feng Cai, School of Basic Medical Sciences, Central South University, Changsha 410013, Hunan, China. Tel: +86 731 82355414; fax: +86 731 82355414; email: cjf_jifeng@163.com

provide rapid and reliable species determination regardless of insect lifecycle stages and are a necessary supplement to morphologic methods. In addition, molecular analysis only requires small tissue samples under relatively insensitive preservation conditions. After testing, the samples may still be used for further morphological study and/or for additional genetic analysis.

The cytochrome oxidase subunit I (COI) region, located in the respiratory chain of the mitochondrial genome, is easy to isolate, has a higher copy number than its nuclear counterparts, and has conserved sequence and structure across taxa (Harvey *et al.*, 2008). In numerous studies covering vast geographic regions, various segments of the COI region, ranging from 229 bp to the entire gene, have been sequenced to differentiate forensically important blow fly species (Wallman & Donnellan, 2001; Harvey *et al.*, 2003a, 2003b; Saigusa *et al.*, 2005; Wallman *et al.*, 2005; Ames *et al.*, 2006; Zhang *et al.*, 2007; Cainé *et al.*, 2009; Desmyter & Gosselin, 2009; Park *et al.*, 2009; Reibe *et al.*, 2009). A 278-bp COI region is a classical genetic marker that has been frequently used to identify forensically important fly species (Wells & Sperling, 1999; Harvey *et al.*, 2003a; Cai *et al.*, 2005; Wells *et al.*, 2007). Consequently, there are abundant authenticated reference DNA sequences in the databases for comparing and identifying species.

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). Many Calliphorid species are the first to locate and oviposit onto corpses, and are among the dominant species found on corpses in China. Previous studies (Harvey *et al.*, 2003a, 2003b, 2008; Wells *et al.*, 2007; Reibe *et al.*, 2009) encountered difficulties in discriminating between closely related Calliphorid species because of paraphyly and polyphyly. This study evaluates the suitability of the 278-bp COI region for identification of blow fly species, under experimental conditions prior to application in Chinese criminal investigations. The 278-bp COI region of 75 Calliphorids was sequenced and deposited in GenBank to expand local databases.

Materials and methods

Insects

Seventy-five sarcosaphagous adult blow flies were collected from 23 locations in 16 provinces of China during the months of June to September for 4 consecutive years (2006 to 2009). Collection information is listed in Table 1. The southernmost location was Fuzhou, Fujian Province (26°05′N, 119°18′E), while the northernmost and westernmost location was Urumqi in Xijiang Autonomous Region (43°45′N, 87°36′E).

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. They were identified by entomologists based on traditional morphological characteristics (Lu & Wu, 2003; Xue & Zhao, 1996). Some relatively cosmopolitan species, such as *Chrysomya megacephala* (Fabricius, 1794), *Chrysomya rufifacies* (Macquart, 1843), and *Calliphora vicina* (Robineau-Desvoidy, 1830), were included in this study. *Protophormia terraenovae* (Robineau-Desvoidy, 1830), *Hemipyrellia ligurriens* (Wiedemann, 1830), and four morphologically similar *Lucilia* species – *L. sericata*, *L. bazini* (Seguy, 1934), *L. porphyrina* (Walker, 1857), *L. caesar* – were also included. *Sarcophaga peregrine* (Robineau-Desvoidy, 1830) of the family Sarcophagidae was chosen as the out-group species. The protocols used in this study had been approved by the constituted Ethics Committee of the Central South University and conforms to the provisions of the Declaration of Helsinki.

DNA extraction

Mitochondrial DNA (mtDNA) from all samples was extracted using the improved grinding tissue DNA extraction method (An *et al.*, 2003). 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.

Polymerase chain reaction (PCR) protocol

The 278-bp fragment of the COI gene from all specimens was amplified using the primers pair C1-J-2495 (5′-CAG CTA CTT TAT GAG CTT TAG G-3′) (sense) and C1-N-2800 (5′-CAT TTC AAG CTG TGT AAG CAT C-3′) (antisense) (Wells & Sperling, 1999; Harvey *et al.*, 2003a). The PCR reaction 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, USA: 4 μL deoxynucleotide triphosphate [dNTP: 1 mmol/mL], 1.0 U *Taq* polymerase, 2.5 μL 10 × buffer [Mg^{2+} 1.5 mmol/L]), 0.25–2.5 μL of each primer

Table 1 Locality and reference data for the newly sequenced specimens.

No.	Species	Location (Latitude [E], Longitude [N])	Preservation method	Accession No.
1	*Chrysomya megacephala* (Fabricius, 1794)	Changsha, Hunan (28°12′, 112°59′)	70% ethanol	FJ746479
2		Linyi, Shandong (35°03′, 118°20′)	70% ethanol	FJ746490
3		Datong, Shanxi (40°06′, 113°17′)	Dried	FJ746464
4		Yongzhou, Hunan (26°13′, 111°37′)	70% ethanol	FJ746488
5		Shijiazhuang, Hebei (38°02′, 114°30′)	70% ethanol	FJ746461
6		Datong, Shanxi (40°06′, 113°17′)	Dried	FJ746478
7		Zhuzhou, Hunan (27°51′, 113°09′)	Dried	FJ746487
8		Chifeng, Inner Mongolia (42°17′, 118°58′)	70% ethanol	FJ746477
9		Zhangjiajie, Hunan (29°08′, 110°29′)	70% ethanol	GU145185
10		Yichun, Jiangxi (27°47′, 114°23′)	Dried	GU145191
11		Shijiazhuang, Hebei (38°02′, 114°30′)	Dried	GU145183
12		Datong, Shanxi (40°06′, 113°17′)	70% ethanol	GU145166
13		Hohhot, Inner Mongolia (40°48′, 111°41′)	70% ethanol	GU145192
14		Changsha, Hunan (28°23′, 112.94′)	70% ethanol	GU145209
15		Yichun, Jiangxi (27°47′, 114°23′)	70% ethanol	GU145213
16		Zhangjiajie, Hunan (29°08′, 110°29′)	70% ethanol	GU145203
17		Shijiazhuang, Hebei (38°02′, 114°30′)	70% ethanol	FJ746460
18		Yongzhou, Hunan (26°13′, 111°37′)	Dried	GU145184
19		Hohhot, Inner Mongolia (40°48′, 111°41′)	Dried	GU145182
20		Datong, Shanxi (40°06′, 113°17′)	70% ethanol	FJ746459
21		Hohhot, Inner Mongolia (40°48′, 111°41′)	70% ethanol	GU145199
22		Changsha, Hunan (28°12′, 112°59′)	Dried	GU145206
23		Yichun, Jiangxi (27°47′, 114°23′)	70% ethanol	GU145181
24		Shijiazhuang, Hebei (38°02′, 114°30′)	70% ethanol	GU145215
25		Zhangjiajie, Hunan (29°08′, 110°29′)	70% ethanol	GU145194
26		Hohhot, Inner Mongolia (40°48′, 111°41′)	70% ethanol	GU145158
27		Beijing (39°55′, 116°24′)	Dried	FJ746485
28		Xinxiang, Henan (35°18′, 113°52′)	70% ethanol	FJ746494
29		Changsha, Hunan (28°12′, 112°59′)	Dried	GU145212
30		Urumqi, Xinjiang (43°45′, 87°36′)	Dried	GU145167
31		Yichun, Jiangxi (27°47′, 114°23′)	Dried	GU145207
32		Xiangxiang, Hunan (27°44′, 112°31′)	70% ethanol	GU145147
33		Yongzhou, Hunan (26°13′, 111°37′)	70% ethanol	GU145160
34		Zhangjiajie, Hunan (29°08′, 110°29′)	70% ethanol	GU145152
35		Urumqi, Xinjiang (43°45′, 87°36′)	70% ethanol	GU145187
36		Xiangxiang, Hunan (27°44′, 112°31′)	70% ethanol	GU145186
37		Changsha, Hunan (28°23′, 112°94′)	70% ethanol	GU145202
38	*Chrysomya rufifacies* (Macquart, 1843)	Yongzhou, Hunan (26°13′, 111°37′)	Dried	GU145144
39		Fuzhou, Fujian (26°05′, 119°18′)	70% ethanol	FJ746466
40		Changsha, Hunan (28°23′, 112°94′)	Dried	GU145170
41		Weifang, Shandong (36°43′, 119°06′)	Dried	GU145193
42	*Protophormia terraenovae* (R-D†, 1830)	Chifeng, Inner Mongolia (42°17′, 118°58′)	70% ethanol	FJ746468

to be continued.

Table 1 Continued.

No.	Species	Location (Latitude [E], Longitude [N])	Preservation method	Accession No.
43	*Hemipyrellia ligurriens* (Wiedemann, 1830)	Quzhou, Zhejiang (28°58′, 118°52′)	Dried	FJ746495
44	*Lucilia sericata* (Meigen, 1826)‡	Yongzhou, Hunan (26°13′, 111°37′)	70% ethanol	GU145205
45		Yinchuan, Ningxia (38°27′, 106°16′)	Dried	FJ746462
46		Yinchuan, Ningxia (38°27′, 106°16′)	Dried	FJ746463
47		Datong, Shanxi (40°06′, 113°17′)	70% ethanol	FJ746458
48		Lanzhou, Gansu (36°04′, 103°51′)	Dried	FJ746474
49		Baotou, Inner Mongolia (40°39′, 109°49′)	Dried	FJ746471
50		Tianjin (39°02′, 117°21′)	70% ethanol	FJ746476
51		Baotou, Inner Mongolia (40°39′, 109°49′)	70% ethanol	FJ746469
52		Xining, Qinghai (36°38′, 101°48′)	70% ethanol	GU145154
53		Shijiazhuang, Hebei (38°02′, 114°30′)	70% ethanol	GU145159
54		Xining, Qinghai (36°38′, 101°48′)	70% ethanol	GU145139
55		Xining, Qinghai (36°38′, 101°48′)	70% ethanol	GU145157
56		Urumqi, Xinjiang (43°45′, 87°36′)	70% ethanol	GU145138
57		Weifang, Shandong (36°71′, 119°12′)	Dried	GU145142
58		Urumqi, Xinjiang (43°45′, 87°36′)	Dried	GU145148
59		Shijiazhuang, Hebei (38°02′, 114°30′)	Dried	GU145143
60		Xining, Qinghai (36°38′, 101°48′)	70% ethanol	GU145149
61		Hohhot, Inner Mongolia (40°48′, 111°41′)	70% ethanol	GU145151
62		Yichun, Jiangxi (27°47′, 114°23′)	70% ethanol	GU145204
63		Xi'an, Shaanxi (34°17′, 108°57′)	Dried	GU145140
64		Urumqi, Xinjiang (43°45′, 87°36′)	Dried	GU145153
65		Changsha, Hunan (28°23′, 112°94′)	70% ethanol	GU145165
66	*Lucilia bazini* (Seguy, 1934)	Zhangjiajie, Hunan (29°08′, 110°29′)	70% ethanol	GU145145
67	*Lucilia porphyrina* (Walker, 1857)	Changsha, Hunan (28°12′, 112°59′)	Dried	GU145146
68	*Lucilia caesar* (Linnaeus, 1758)§	Tianjin (39°02′, 117°21′)	70% ethanol	FJ746475
69		Linyi, Shangdong (35°03′, 118°20′)	Dried	FJ746489
70		Beijing (39°55′, 116°24′)	70% ethanol	FJ746483
71		Yichun, Jiangxi (27°47′, 114°23′)	70% ethanol	GU145201
72		Xinxiang, Henan (35°18′, 113°52′)	Dried	FJ746493
73		Xi'an, Shaanxi (34°17′, 108°57′)	70% ethanol	GU145150
74	*Calliphora vicina* (R-D†, 1830)	Xining, Qinghai (36°38′, 101°48′)	70% ethanol	GU145168
75		Hohhot, Inner Mongolia (40°48′, 111°41′)	70% ethanol	GU145169
76	*Sarcophaga peregrine* (R-D†, 1830)	Shijiazhuang, Hebei (38°02′, 114°30′)	Dried	GU145176
77		Yongzhou, Hunan (26°13′, 111°37′)	70% ethanol	GU145177

†R-D, Robineau-Desvoidy.

‡For *L. sericata*, specimen 44 belonged to clade 1, specimens 45–48 belonged to clade 2, 49–51 belonged to clade 3, and 52–65 belonged to clade 4.

§For *L. caesar*, specimens 68–70 belonged to clade 1, 71–72 belonged to clade 2, and specimen 73 belonged to clade 3.

(10 μmol/L), and Nuclease-Free Water (Promega) added to a total volume of 25 μL.

Amplifications were performed in a thermocycler (Perkin-Elmer 9600) programmed with the following parameters: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s, and a final elongation at 72°C for 5 min.

Sequencing

Vertical non-denaturing polyacrylamide gel electrophoresis was used to isolate PCR products, which were then purified using a QiaQuick PCR Purification Kit (Qiagen, Germantown, MD, USA). Column 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, USA) 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).

COI sequencing 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.ddbj.nig.ac.Jp/E-mail/clastalw-e.html). The obtained sequences have been deposited in GenBank by Sequin (http://www.ncbi.nlm.nih.Gov/equin/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 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) function.

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). Evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 500 replicates was inferred to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). 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 (Tamura *et al.*, 2004) 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. Neighbor-joining analysis was performed using the Tamura-Nei model of substitution and bootstrapping ($n = 500$) through MEGA4.

Results

Morphological identification of specimens

All samples were identified by entomologists into nine distinct morphological types (species) and were assigned to three subfamilies (Chrysomyinae, Luciliinae, and Calliphorinae) using traditional morphological characteristics. Each of the five species groups (*Ch. megacephala*, *Ch. rufifacies*, *L. sericata*, *C. vicina* and *L. caesar*) had more than one representative; whereas *L. bazini*, *L. porphyrina*, *P. terraenovae* and *H. ligurriens* were singletons. The taxonomic relationships between the 75 blow flies and other relevant species are shown in Table 2. Previous studies about these species and regions of COI are also listed in Table 2.

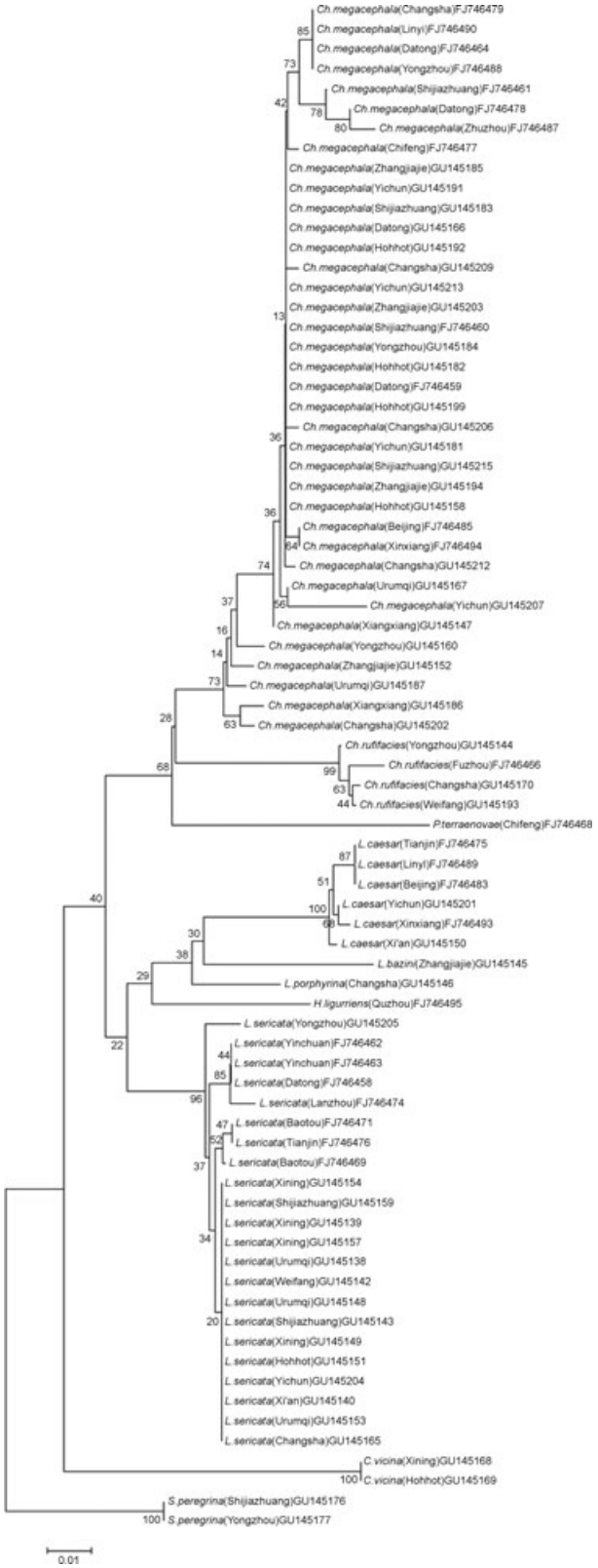
Phylogenetic analysis

Phylogenetic analysis was used to study the relationships between different groups of organisms based on their similarities and differences in selected characteristics. There were 278 aligned sites in the analysis of the 77 COI sequences (Fig. 1). Two specimens of the outgroup species *S. peregrine* were clustered together with a supporting bootstrap of 100%, and they were clearly separate from the Calliphorids in the NJ tree (Fig. 1). At the species level, five distinct congeneric and monophyletic clusters (*Ch. megacephala* cluster, *Ch. rufifacies* cluster, *C. vicina* cluster, *L. caesar* cluster and *L. sericata* cluster) were well-formed with high bootstrap values (≥96%), except for *Ch. megacephala* at 73%. At the genus and subfamily levels, all Calliphorid species were correctly assigned to their appropriate subfamilies (Chrysomyinae, Luciliinae and Calliphorinae). Two subfamilial mitochondrial networks, namely Chrysomyinae and Luciliinae, were formed but with relatively weak supporting values of 22% and 68%, respectively; whereas there was only one species in subfamily Calliphorinae. Three *Lucilia* species (*L. caesar*, *L. bazini* and *L. porphyrina*) clustered together with a weak bootstrap value of 38%. *Ch. megacephala* clustered with its congeneric species *Ch. rufifacies* but with a weak supporting value (28%).

Table 2 Taxonomy of sarcosaphagous fly specimens in this study and related studies using COI regions.

Family	Subfamily	Genus	Species	No.†	References
Calliphoridae	Chrysomyinae	*Chrysomya*	*Ch. megacephala*	1–37	Chen *et al.* (2009) used 1 120 bp; Harvey *et al.* (2003a) used 278 bp; Harvey *et al.* (2003b) used 1 167 bp; Harvey *et al.* (2008) used 1 167 bp; Wang *et al.* (2007) used 348 bp; Wells and Williams (2007) used several regions.
			Ch. rufifacies	38–41	Harvey *et al.* (2003a) used 278 bp; Harvey *et al.* (2003b) used 1 167 bp; Harvey *et al.* (2008) used 1 167 bp; Wallman *et al.* (2005) used 822 bp; Wang *et al.* (2007) used 348 bp; Wells *et al.* (2007) used 1 545 bp; Wells and Williams (2007) used several regions.
		Protophormia	*P. terraenovae*	42	Desmyter and Gosselin used 304 bp; Reibe *et al.* (2009) used 229 bp; Wells and Williams (2007) used several regions.
	Luciliinae	*Hemipyrellia*	*H. ligurriens*	43	Park *et al.* (2009) used 1 539 bp; Wells *et al.* (2007) used 1 545 bp; Wallman *et al.* (2005) used 822 bp.
		Lucilia	*L. sericata*	44–65	Chen *et al.* (2009) used 1 120 bp; Desmyter and Gosselin (2009) used 304 bp; Reibe *et al.* (2009) used 229 bp; Harvey *et al.* (2003a) used 278 bp; Harvey *et al.* (2003b) used 1 167 bp; Harvey *et al.* (2008) used 1 167 bp; Saigusa *et al.* (2005) used 304 bp; Saigusa *et al.* (2009) used 304 bp; Wallman *et al.* (2005) used 822 bp; Wells *et al.* (2007) used 1 545 bp; Wang *et al.* (2007) used 348 bp.
			L. bazini	66	Wells *et al.* (2007) used 1 545 bp.
			L. porphyrina	67	Wallman *et al.* (2005) used 822 bp; Wells *et al.* (2007) used 1 545 bp.
			L. caesar	68–73	Cainé *et al.* (2009) used several regions; Park *et al.* (2009) used 1 539 bp; Reibe *et al.* (2009) used 229 bp; Wells *et al.* (2007) used 1 545 bp.
	Calliphorinae	*Calliphora*	*C. vicina*	74–76	Ames *et al.* (2006) used approximately 400 bp; Cainé *et al.* (2009) used several regions; Chen *et al.* (2009) used 1 120 bp; Reibe *et al.* (2009) used 229 bp; Harvey *et al.* (2008) used 1 167bp; Wallman *et al.* (2005) used 822 bp.

†Specimen number is in the same order as that in Table 1.



COI sequence divergence

Table 3 shows that all the maximum intraspecific divergences were less than 1.2%, except for *Ch. megacephala* at 4.3% and *L. sericata* at 2.1%. Levels of interspecific divergences varied from 4.5% to 15.4%. The lowest interspecific divergence (4.5%) was observed between the congeneric species *L. sericata* and *L. porphyrina*.

Geographic variation

A country-wide geographic study of *L. sericata*, *L. caesar* and *Ch. megacephala* was executed at the species level. Both the inter-site and intra-site variations of *Ch. megacephala* varied from 0.0% to 2.0% (data not shown), indicating that there was no obvious geographic variation. For *L. sericata*, four different clades were formed while *L. caesar* had three clades (Fig. 1). In Table 4, the maximum intra-cladistic specific variations of either *L. sericata* or *L. caesar* were relatively lower than their corresponding inter-cladistic specific variations. Specifically, the maximum intra-cladistic specific variation in clade 2 of *L. sericata* was 0.6%, while the inter-cladistic specific variation between species in clade 2 and in each of the other three clades was no more than 0.7%. Within *L. caesar*, each maximum intra-cladistic specific variation (below 0.3%) was also lower than the corresponding inter-cladistic specific variations (0.4%–0.9%). These findings indicate some intraspecific geographic variations in *L. sericata* and *L. caesar*.

Discussion

All the 77 forensically important sarcosaphagous adult fly specimens were obtained from 23 sites of 16 provinces in China during June to September of 4 consecutive years (2006 to 2009). DNA of all samples were successfully extracted using the improved grinding tissue DNA extraction method, and both sample preservation methods (70% ethanol and drying) used were efficient.

Fig. 1 Neighbor-joining tree displaying evolutionary relationships of 77 taxa. *S. peregrine* was chosen as the out-group. Bootstrap values indicate support for nodes. The bar indicates 0.01 substitutions per site. †*Ch. megacephala*, *Ch. rufifacies*, *P. terraenovae*, *L. caesar*, *L. bazini*, *L. porphyrina*, *H. ligurriens*, *L. sericata*, *C. vicina* are abbreviation of *Chrysomya megacephala*, *Chrysomya rufifacies*, *Protophormia terraenovae*, *Lucilia caesar*, *Lucilia bazini*, *Lucilia porphyrina*, *Hemipyrellia ligurriens*, *Lucilia sericata*, *Calliphora vicina* respectively.

Table 3 Calculated intraspecific and interspecific divergences expressed as percentage of total 278 bp of COI using neighbor-joining approach with Tamura-Nei model of substitution.

No.	Species	No.†	Range‡	1	2	3	4	5	6	7	8	9
1	*L. sericata*	22	0.0–2.1	–								
2	*L. bazini*	1	–	7.2	–							
3	*L. porphyrina*	1	–	4.5	6.5	–						
4	*L. caesar*	6	0.0–0.9	7.4	7.1	5.2	–					
5	*H. ligurriens*	1	–	7.0	7.9	6.6	8.5	–				
6	*Ch. rufifacies*	4	0.3–1.2	9.2	9.6	9.8	12.0	9.9	–			
7	*Ch. megacephala*	36	0.0–4.3	6.9	10.6	8.6	9.3	8.8	6.9	–		
8	*P. terraenovae*	1	–	10.5	11.2	11.2	9.7	11.6	10.2	8.7	–	
9	*C. vicina*	2	0.0	9.8	12.1	11.5	10.7	11.0	14.7	11.9	15.4	–

†Number of specimens.

‡Range of intraspecific variations (minimum to maximum value).

Species and group identification

All specimens were properly classified into their respective species groups and the results of molecular blow fly species identification correlated with the initial morphological identification. Although several studies considered some *Lucilia* species pairs difficult to differentiate (Stevens & Wall, 2001; Stevens *et al.*, 2002; Wallman *et al.*, 2005; Ying *et al.*, 2007; Wells *et al.*, 2007; Harvey *et al.*, 2008; Reibe *et al.*, 2009; Tourle *et al.*, 2009), the topology (Fig. 1) and the distinct intraspecific versus interspecific divergence (Table 3) suggests good discrimination among the four morphologically similar *Lucilia* species (*L. bazini*, *L. porphyrina*, *L. caesar* and *L. sericata*). The taxonomic relationships between *Lucilia* species concur with previous studies (Stevens & Wall, 1996; Stevens & Wall, 2001), and confirm the monophyletic *L. bazini/ L. porphyrina/ L. caesar* cluster and their separation from *L. sericata*. *H. ligurriens* was unexpectedly embedded within the *Lucilia* cluster; similar findings have also been reported by Wells *et al.* (2007) using a 1 545-bp COI region and by Park *et al.* (2009) using the full length of COI region as the molecular marker.

Evaluation of differences in intra- versus interspecific variation has been suggested as a potentially useful criterion for species identification (Wells & Sperling, 2001; Amendt *et al.*, 2004). The maximum intraspecific divergences of *Ch. megacephala* and *L. sericata* of 4.3% and 2.1%, respectively, were not much higher compared with the study by Harvey *et al.* (2008) using a 1 167-bp COI region, which reported the worldwide variability of *Ch. megacephala* and *L. sericata* at 0.34% and 0.26% respectively. Furthermore, Reibe *et al.* (2009) found only 0.5% variability in German *L. sericata* using a 229-bp COI region. Since they are carrion-breeding species, they have wide geographic distributions that partly explain the relatively large intraspecific divergence of the two species. In addition, their variable behavior (e.g. hybridization) may also lead to high intraspecific genetic variations. Meanwhile, the possibility of misidentification of a specimen

Table 4 Intra-cladistic versus inter-cladistic specific variations in *L. sericata* and *L. caesar*.

No.	Species clade	No.†	Range‡	1	2	3	4	5	6	7
1	*L. sericata* clade 1	1	–	–		–				
2	*L. sericata* clade 2	4	0.0–0.6	1.6	–					
3	*L. sericata* clade 3	3	0.0–0.3	1.3	1.1	–				
4	*L. sericata* clade 4	14	0.0–0.0	0.9	0.7	0.4	–			
5	*L. caesar* clade 1	3	0.0–0.0					–		
6	*L. caesar* clade 2	2	0.3					0.7	–	
7	*L. caesar* clade 3	1	–					0.9	0.4	–

†Number of specimens.

‡Range of intra-cladistic specific variations (minimum to maximum value).

still exists. Furthermore, the use of a relatively short COI region (278 bp) for analysis may also magnify the variations between different sequences. All these may be responsible for the low bootstrap values in the *Ch. megacephala* and *L. sericata* species cluster. Although the intraspecific divergences of the two species were relatively high, the corresponding interspecific divergences were higher (Table 3) at 6.9% and 5.5%, respectively. This indicates that they can still be correctly differentiated from other species. Furthermore, the range of divergence in Table 3 falls within ranges in some previous reports (Desmyter & Gosselin, 2009; Park *et al.*, 2009; Reibe *et al.*, 2009); the clear differences between the threshold levels of intraspecific versus interspecific divergence enable unambiguous differentiation of the nine forensically important blow fly species.

Geographic analysis

In general, the insect succession that occurs during corpse decomposition is related to season, temperature, humidity and type of habitat (Shi *et al.*, 2009). Geographic and environmental conditions influence the fauna in a particular area, especially between cities and provinces. Geographic variations within forensically relevant species have been reported for *L. sericata*, *Phormia regina* (Meigen, 1826), *Lucilia cuprina* (Wiedemann, 1830), and *Ch. rufifacies* (Harvey *et al.*, 2003a, 2008; Cai *et al.*, 2006; Wallman *et al.*, 2005; Wells *et al.*, 2007; Desmyter & Gosselin, 2009). However, Picard and Wells (2010) found a negligible correlation between geographic and genetic distances in North American *L. sericata* through a Mantel test ($r_2 = 0.006\,3$, $P = 0.02$).

In the present study, the tree topology (Fig. 1) and intra- versus inter-cladistic specific variations (Table 4) reflect differences between the clades of *L. sericata* and *L. caesar*. In most cases, these geographic variations can be explained by the ecological and geographical conditions of the collection locations, such that members of the same clade were recovered from similar environments and members of different clades were from environmentally different locales. However, in this study, specimens of each of the two species in the same clade were recovered from locations that were far from each other. These findings may be attributed to various factors: (i) samples in the same clade could have spread to distant sites through human activities, since most carrion-breeding flies are synanthropic; and (ii) some cryptic species and subspecies, or even misidentified species were not eliminated from the analysis and are skewing the data. For *Ch. megacephala*, multiple monotypes have been observed at a single location and at multiple locations (Fig. 1); this distribution supports the results mentioned above, since there is no obvious geographic variation for *Ch. megacephala*.

Implications for future work

Species compositions have their own local characteristics (Vanin *et al.*, 2008). As such, ecological succession data obtained from corpses at one geographic location do not necessarily apply to other locations (Wang *et al.*, 2008). This study is the first step toward developing a fast, accurate and easily accessible mtDNA-based biomolecular species identification system, which together with traditional morphological methods and correlative ecological succession data, would help identify forensically important sarcosaphagous insects. A large number of specimens across vast regional distributions must be collected and correlated with ecological succession data to enrich local databases for further large-scale geographic analysis. Morphologic analyses are also needed to discover more distinct morphological characteristics and to eliminate mistakes in species identification.

The length of nucleotide sequences used in this study is still relatively short (278 base pairs) for reliable phylogenetic analysis and some positions of taxa in the phylogenetic tree (Fig. 1) did not concur with current taxonomic treatment of Calliphoridae. Therefore, more extensive sequencing of the COI gene and other mitochondrial and nuclear DNA sequences must be executed to solve these problems. According to Amendt *et al.* (2004), the combined analysis of mtDNA fragments is a more accurate approach for Dipteral species identification than single COI fragment analysis. In a future work, simultaneous sequencing of a 272-bp COI region, a 250-bp region of mtDNA from the 16S ribosomal DNA gene (16S rDNA), and a 246-bp region of COII from some of the specimens studied here will be performed. Further combined analysis (e.g. COI + COII + 16Sr + morphology + ecology) would be carried out for fly species identification, geographic distribution analysis and taxonomic classification.

Conclusion

Analysis of the 278-bp region of the COI gene in 75 Calliphorid specimens collected from 23 locations in China illustrate the enormous potential of the COI region as an economical discriminatory tool in forensic entomological investigations. All the blow fly species, especially four morphologically similar *Lucilia* species, were well

discriminated. Geographic variations were observed for *L. sericata* and *L. caesar*, although explanations for their development remain unexplored. Some taxonomic relationships among genera failed to match traditional taxonomic classification. Further studies are required to resolve these discrepancies.

Acknowledgments

This study was funded by the National Natural Science Foundation of China (NSFC, No. 30672354) and National University Student Innovation Test Plan (NMOE, YC09139, YC10107, YC10117). We thank all the members of the forensic community from the Central South University for their help in sample collection. We are also grateful to Professors Song Dong-Bao and Huang Guo-Hua, Ph.D. (Plant Protection Department, College of Bio-safety Science and Technology, Hunan Agricultural University) for the identification of insects.

References

Alessandrini, F., Mazzanti, M., Onofri, V., Turchi, C. and Tagliabracci, A. (2008) MtDNA analysis for genetic identification of forensically important insects. *Forensic Science International: Genetics Supplement Series*, 1, 584–585.

Amendt, J., Krettek, R. and Zehner, R. (2004) Forensic entomology. *Naturwissenschaften*, 91, 51–65.

Ames, C., Turner, B. and Daniel, B. (2006) The use of mitochondrial cytochrome oxidase I gene (COI) to differentiate two UK blowfly species – *Calliphora vicina* and *Calliphora vomitoria*. *Forensic Science International*, 164, 179–182.

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

Anderson, G.S. (2004) Determining time of death using blowfly eggs in the early postmortem interval. *International Journal of Legal Medicine*, 118, 240–241.

Cai, J.F., Liu, M., Ying, B.W., Deng, R.L., Dong, J.G., Zhang, L., Tao, T., Pan, H.F., Yan, H.T. and Liao, Z.G. (2005) The availability of mitochondrial DNA cytochrome oxidase I gene for the distinction of forensically important flies in China. *Acta Entomologica Sinica*, 48, 380–385.

Cai, J.F., Lan, L.M., Chen, Y.Q., Wang, Z.X., Chang, Y.F., Liang, W.B., Dong, J.G. and Liao, Z.G. (2006) Relationship between COII gene of mtDNA of *Lucilia sericata* and latitude interval. *Journal of Forensic Medicine*, 22, 401–403.

Cainé, L., Corte Real, F., Saloña-Bordas, M., Martínez de Pancorbo, M., Lima, G., Magalhães, T. and Pinheiro, F. (2009) DNA typing of Diptera collected from human corpses in Portugal. *Forensic Science International*, 184, e21–e23.

Chen, Q., Bai, J., Liu, L., Lin, H.B., Tang, H., Zhao, W., Zhao, H.Z., Yan, J.W., Liu, Y.C. and Hu, S.N. (2009) Sequential analysis of mitochondriaI COI gene for seven common sarcosaphagous flies (Diptera) in Beijing and the establishment of their DNA barcodes. *Acta Entomologica Sinica*, 52, 202–209

Desmyter, S. and Gosselin, M. (2009) COI sequence variability between Chrysomyinae of forensic interest. *Forensic Science International-Genetics*, 3, 89–95.

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

Grassberger, M. and Frank, C. (2004) Initial study of arthropod succession on pig carrion in a central European urban habitat. *Journal of Medical Entomology*, 41, 511–523.

Harvey, M.L., Dadour, I.R. and Gaudieri, S. (2003a) Mitochondrial DNA cytochrome oxidase I gene: potential for distinction between immature stages of some forensically important fly species (Diptera) in western Australia. *Forensic Science International*, 131, 134–139.

Harvey, M.L., Mansell, M.W., Villet, M.H. and Dadour, I.R. (2003b) Molecular identification of some forensically important blowflies of southern Africa and Australia. *Medical and Veterinary Entomology*, 17, 363–369.

Harvey, M.L., Gaudieri, S., Villet, M.H. and Dadour, I.R. (2008) A global study of forensically significant calliphorids: implications for identification. *Forensic Science International*, 177, 66–76.

Lu, B.L. and Wu, H.Y. (2003) *Classification and Identification of Important Medical Insects of China*. Henan Science and Technology Publishing House, Zhengzhou. (in Chinese).

Park, S.H., Zhang, Y., Piao, H., Yu, D.H., Jeong, H.J., Yoo, G.Y., Chung, U., Jo, T.H. and Hwang, J.J. (2009) Use of cytochrome c oxidase subunit I (COI) nucleotide sequences for identification of the Korean Luciliinae fly species (Diptera: Calliphoridae) in forensic investigations. *Journal of Korean Medical Science*, 24, 1058–1063.

Picard, C.J. and Wells, J.D. (2010) The population genetic structure of North American *Lucilia sericata* (Diptera: Calliphoridae), and the utility of genetic assignment methods for reconstruction of postmortem corpse relocation. *Forensic Science International*, 195, 63–67.

Reibe, S., Schmitz, J. and Madea, B. (2009) Molecular identification of forensically important blowfly species (Diptera: Calliphoridae) from Germany. *Parasitology Research*, 175, 17–22.

Saigusa, K., Matsumasa, M., Yashima, Y., Takamiya, M. and Aoki, Y. (2009) Practical applications of molecular biological species identification of forensically important flies. *Legal Medicine*, 11, S344–S347.

Saigusa, K., Takamiya, M. and Aoki, Y. (2005) Species identification of the forensically important flies in Iwate prefecture,

Japan based on mitochondrial cytochrome oxidase gene subunit I (COI) sequences. *Legal Medicine (Tokyo, Japan)*, 7, 175–178.

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

Shi, Y.W., Liu X.S., Wang, H.Y. and Zhang, R.J. (2009) Seasonality of insect succession on exposed rabbit carrion in Guangzhou, China. *Insect Science*, 16, 425–439.

Stevens, J. and Wall, R. (1996) Classification of the genus *Lucilia* (Diptera: Calliphoridae): a preliminary parsimony analysis. *Journal of Natural History*, 30, 1087–1094.

Stevens, J. and Wall, R. (2001) Genetic relationships between blowflies (Calliphoridae) of forensic importance. *Forensic Science International*, 120, 116–123.

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

Tamura, K., Nei, M. and 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. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution*, 24, 1596–1599.

Tourle, R., Downie, D.A. and Villet, M.H. (2009) Flies in the ointment: a morphological and molecular comparison of *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae) in South Africa. *Medical and Veterinary Entomology*, 23, 6–14.

Vanin, S., Tasinato, P., Ducolin, G., Terranova, C., Zancaner, S., Montisci, M., Ferrara, S.D. and Turchetto, M. (2008) Use of *Lucilia* species for forensic investigations in Southern Europe. *Forensic Science International*, 177, 37–41.

Wallman, J.F. and Donnellan, S.C. (2001) The utility of mitochondrial DNA sequences for the identification of forensically important blowflies (Diptera: Calliphoridae) in southeastern Australia. *Forensic Science International*, 120, 60–67.

Wallman, J.F., Leys, R. and Hogendoorn, K. (2005) Molecular systematics of Australian carrion-breeding blowflies (Diptera: Calliphoridae) based on mitochondrial DNA. *Invertebrate Systematics*, 19, 1–15.

Wang, J.F., Li, Z.G, Chen, Y.C., Chen, Q.S. and Yin, X.H. (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.

Wang, J.F., Yin, X.H. and Chen, Y.C. (2007) Molecular identification of five common species of necrophagous flies in China. *Acta Entomologica Sinica*, 50, 423–428.

Wells, J.D., and Sperling, F.A.H. (1999) Molecular phylogeny of *Chrysomya albiceps* and *C. rufifacies* (Diptera: Calliphoridae). *Journal of Medical Entomology*, 36, 222–226.

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

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

Wells, J.D. and 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.

Xue, W.Q. and Zhao, J.M. (1996) *Flies of China*. Liaoning Science and Technology Press, Shenyang. (in Chinese).

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

Zhang, Y., Park, S.H., Yu, D.H., Yu, G.Y., Jung, H.J., Jo, T.H. and Hwang, J.J. (2007) Identification blow fly species in Korea by mitochondrial DNA 'Barcodes'. *Journal of Korean Medical Science*, 31, 51–58.

Accepted July 13, 2010