

Comparative Analysis of Molecular Identification and Wing Morphometrics of Forensically Important Blow Flies in China

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Abstract

Background: Correct species identification is the most crucial step in applying entomological evidence to estimate the postmortem interval (PMI) since death of decomposed corpses. Wing morphometrics have been proposed in species classification as an alternative method of traditional morphology and molecular approaches. However, so far, this method has not been applied to the identification of Chinese Calliphoridae and few studies compare the two identification methods.

Methods: We used landmark-based geometric morphometrics of wings to identify nine medically and forensically important blow fly species of China. 270 specimens representing nine species and eight genera were sampled, 18 landmarks on the right wing were measured and analyzed using canonical variates analysis and discriminant function analysis. Then, a cross-validation test was used to evaluate reliability of the method. Moreover, in order to further assess the validity of this method, molecular identification is used for comparative analysis. Eighty sequences of cytochrome c oxidase subunit I (COI) of Calliphoridae isolated from different countries were downloaded from Genbank, including the data previously submitted by our team.

Results: Different species and genera can be well separated through morphometric analysis with an overall classification accuracy of 80~100%, but discrimination between sexes was less effective. The results indicated that the discriminative efficiency of the two methods is almost identical.

Conclusions: Wing morphometrics can be used as a complementary method of molecular identification for the geographical location and gender identification of certain species as a simple and cheap method.

Background

As an important forensic tool, forensic entomology, the interpretation of insect evidence in legal investigations, has been applied prevalently and rapidly to many countries in the early 21st century [1]. Correct identification of insect specimens collected from corpses or a crime scene is a crucial step of forensic entomology, which can provide critical information for forensic cases, including estimation of the minimum postmortem interval (PMI_{min}) to solve cases involving death [2, 3]. Blow fly (Diptera: Calliphoridae) is usually the first colonizers of corpses, and also the most common insect related to animal and human corpses, and therefore become the focus of forensic entomological research[4]. Many of them are widely distributed around the world while others live in certain geographic regions. In general, the flesh flies can be identified by morphological method [5, 6, 7]. However, due to the high complexity of the structure of male reproductive organs, it is difficult for non-taxonomists to recognize them.

As an alternative to traditional morphological recognition methods, DNA-based analysis identification has been developed rapidly and widely used in many counties all over the world as a reliable routine tool in forensic entomology, which only requires a small amount of sample at any life cycle stage [8]. Among commonly applied genetic markers, mitochondrial cytochrome c oxidase subunit I (COI) has been extensively used because of its high copy number, high mutation rate, and haploid maternal inheritance [9]. Many studies have proved the robustness of COI as a marker for fly species discrimination [10]. Nevertheless, there is no significant difference in the usage of short fragments or even the entire sequence of COI [11]. DNA barcoding is based on a standardized small segment fragment sequence of cytochrome oxidase subunit 1 (COI) called "DNA barcode" and is widely used in public databases, such as GenBank and Bold (life data barcode). Thus, DNA barcoding is also widely used to identify fly species [12]. In addition, many studies have proved the effectiveness of COI barcodes for the identification of many

species of Calliphoridae [13, 14]. Meanwhile, in GenBank, there are abundant molecular data about Calliphora, sequences of forensic important Calliphora have been published and uploaded online from different regions of the world, such as South Africa, the Caribbean Region, Southern European, the United States and Korea [15, 16, 17, 18], making it possible to systematically study the sequence of Calliphora around the world. However, the molecular identification is a destructive approach for sample materials. Sequencing has relative higher cost and sequence analysis might be problematic in identification of closely related species [19, 20]. Therefore, other effective alternative methods were needed.

Besides DNA identification, geometric morphometrics is a method by using a series of reference points or landmarks that make the morphological comparison of any object structure possible, removing irrelevant information such as the position and orientation of the specimens, considering only their shape [21]. It is a quantitative study of the size, shape and shape changes of species and their covariation with other biotic or abiotic factors [22]. As an alternative approach, the use of landmark-based geometric morphometric analysis of insect wings has shown to be a valuable tool in many studies following early work by Brown [23]. Wing morphometrics has been increasingly popular in studies of Diptera, demonstrating the species identification value in Syrphidae [24] and forensically important families like Muscidae [25] and Sarcophagidae [26]. It has been proved that wing morphometrics can be used for the identification of necrophagous Calliphoridae from Thailand [27] and Europe [28], and distinguish different genus and species of Calliphoridae, *Cochliomyia* [29], *Lucilia* [23, 30] and *Chrysomya* [29, 31]. In addition, geometric morphometric analysis can be used to distinguish variability between geographical populations [29, 32], seasonal morphs [33] or sexes [26, 34] among the same species, and recently it is used to identify Piophilidae (Diptera) for forensic purpose [35]. All of these shows that the analysis of wing morphometric is a very useful identification method.

Up to now, morphometric analysis has not been widely used in the species of Chinese Calliphoridae. In this study, we apply wing morphometric for the identification of necrophagous flies in China at genus and species levels, evaluating the effect of allometric growth on species identification. As female flies are more common and difficult to identify in the forensic field, the differences between male and female wings of nine species of flies were also investigated. In addition, we compared gene sequences of Calliphora from different cities of China with those from different countries and evaluate the reliability of the two types of methods for the identification of forensically relevant species of Calliphoridae. Therefore, this study examined the potential of these tools for specimen identification and concluded that wing morphometrics could be a simple and user-friendly method for research and practical application of forensic entomology.

Methods

Specimen collection

For wing morphometrics analysis, materials for the present study were collected from different places of China. Adult flies, both males and females, were collected directly with a handheld fly net or lured to slightly decomposed pig cadavers placed in a field. All specimens were identified according to Gregor *et al.* and then preserved in ethanol. A total of 270 specimens representing 8 genera and 9 species were used in this study: *Triceratopyga calliphoroides* (Rohdendorf, 1931), *Calliphora vomitoria* (Linnaeus, 1758), *Chrysomya megacephala* (Fabricius, 1794), *Chrysomya rufifacies* (Macquart, 1843), *Calliphora vicina* (Robineau-Desvoidy, 1830), *Aldrichina grahami* (Aldrich, 1930), *Hemipyrellia ligurriens* (Wiedemann, 1830), *Lucilia sericata* (Meigen, 1826), *Protophormia*

terraenovae (Robineau-Desvoidy, 1830). Each species included 15 males and 15 females. Hence, 9 species included in this study involved most of the taxa that are of medical and forensic importance (Table 1).

Table 1
Samples List of Chinese blow fly used in this study

Subfamily	Species	Species code	Collection site	Province	No. of specimens		
					Males	Females	Total
<i>Calliphorinae</i>	<i>A.grahami</i>	AG	Xishuangbannna	Yunnan	3	2	5
			Guiyang	Guizhou	4	5	9
			Shenzhen	Guangdong	6	5	11
			Chongqing	Chongqing	2	3	5
			total		15	15	30
	<i>C.vicina</i>	CVI	Lhasa	Tibet	5	5	10
			Urumchi	Xinjiang	8	7	15
			Guiyang	Guizhou	1	1	2
			Xinyang	Henan	1	2	3
			total		15	15	30
	<i>C.vomitorea</i>	CVO	Lhasa	Tibet	9	12	21
			Jining	Shandong	5	3	8
			Lundazi	Tibet	1	-	1
			total		15	15	30
	<i>T.calliphoroides</i>	TC	Qufu	Shandong	6	8	14
			Beijing	Beijing	3	4	7
Jining			Shandong	4	3	7	
Gunzhou			Shandong	2	-	2	
total				15	15	30	
<i>Chrysomyinae</i>	<i>Ch.megacephala</i>	CM	Changsha	Hunan	10	12	22
			Guilin	Guangxi	5	3	8
			total		15	15	30
	<i>Ch.rufifacies</i>	CR	Shenzhen	Guangdong	6	8	14
			Guiyang	Guizhou	3	5	8
			Chongqing	Chongqing	6	2	8
			total		15	15	30
	<i>P.terraenovae</i>	PT	Lhasa	Tibet	15	15	30
			total		15	15	30
	<i>Luciliinae</i>	<i>H.ligurriens</i>	HL	Changsha	Hunan	3	5

		Xishuangbanna	Yunnan	6	8	14
		Guiyang	Guizhou	6	2	8
		total		15	15	30
<i>L. sericata</i>	LS	Lhasa	Tibet	13	15	28
		Guiyang	Guizhou	2	-	2
		total		15	15	30

Molecular Analysis

COI gene sequences are obtained from Genbank, and the length was set more than 650 bp to contain the region of COI barcode. Then, alignment and phylogenetic were performed in MEGA 10.0.5 (Center for Evolutionary Medicine and Informatics) [36] yielding a total data set of 80 COI sequences (Table S1). Consensus sequences were aligned using ClustalW under default parameters. The neighbor-joining (NJ) tree construction and genetic divergences calculation were conducted using the uncorrected pairwise distance model in MEGA version 10.0.5 [36]. The constructed tree was generated with the Tamura 3-parameter model, and 500 bootstrap replicates were used to assess the reliability of the tree [37]. Genetic distances were calculated by the Kimura 2-parameter (K2P) model in MEGA version 10.0.5 [36].

Wing Morphometrics

Shape variation of genera and species

At the genera level, the CVA revealed seven canonical variates, and the first two canonical variates (Fig. S1) explained 75% of the total variation (CV1 = 52, CV2 = 23). The scatter diagram from CV1 and CV2 (Fig. S1) showed three clearly separated clusters. The first cluster were specimens of genera *Hemipyrellia*, which was form a distinct group separated from other genera. The second cluster was genera *Achoetandrus* and *Chrysomya*. The third cluster included specimens of genera *Lucilia*, *Calliphora*, *Aldrichina*, *Triceratopyga* and *Protophormia*. The Mahalanobis distances acquired from the pairwise comparisons of all eight genera indicated highly significant differences (permutation test with 10,000 rounds in MorphoJ: $P < 0.0001$), ranging from 4.0299 (*Calliphora* and *Aldrichina*) to 29.9547 (*Achoetandrus* and *Protophormia*) (Table S2). The percentages of correctly classified specimens obtained from the cross-validation test ranged from 80–100.0% (Table S3).

At the species level, the CVA revealed eight canonical variates, the first two canonical variates (Fig. 3a) accounted 80 of the total variation (CV1 = 59, CV2 = 21). The scatter diagram from CV1 and CV2 (Fig. 3a) showed that there are three clearly separated clusters. The first cluster were specimens of species *H. igurriens*; the second cluster included species *Ch. egacephala* and *Ch. ufifacies*; the third cluster consisted of *Pr. erraenovae*, *T. alliphoroides*, *A. rahami*, *C. vicina*, *C. Vomitoria* and *L. sericata*. The Mahalanobis distances acquired from the pairwise comparisons of all nine species indicated highly significant differences (permutation test with 10,000 rounds in MorphoJ: $P < 0.0001$), ranging from 4.9238 (*A. grahami* and *C. vicina*) to 31.6453 (*T. calliphoroides* and *Ch. rufifacies*) (Table 3). Visualized shape changes along CV1 axis were found with landmarks 2, 3, 4, 5, 6, 9, 10, 16, 17,

while shape changes along CV2 axis were most clear using landmarks 2, 3, 4, 5, 6, 7, 10 (Fig. 3b). The percentages of correctly classified specimens acquired from the cross-validation test ranged from 80–100% (Table 4).

Table 3
Difference in wing shapes of nine Calliphoridae species with canonical variate analysis(CVA).

	AG	CVO	CVI	CM	CR	HL	LS	PT	TC
AG	-	0.0462	0.0181	0.0775	0.0949	0.0414	0.0310	0.0397	0.0218
CVO	9.5639	-	0.0409	0.0437	0.0587	0.0627	0.0469	0.0691	0.0555
CVI	4.9238	6.2706	-	0.0766	0.0931	0.0487	0.0407	0.0485	0.0229
CM	19.6195	10.2053	15.9017	-	0.0288	0.0820	0.0661	0.0896	0.0882
CR	26.8853	11.7193	14.9944	6.4564	-	0.0960	0.0812	0.1060	0.1058
HL	10.6829	10.3548	11.0167	10.1122	12.5507	-	0.0285	0.0665	0.0593
LS	11.8235	11.4345	12.5332	14.8977	16.0697	10.1582	-	0.0519	0.0494
PT	10.9679	13.8355	12.8309	21.9797	29.9547	20.8557	15.3188	-	0.0364
TC	6.6546	11.911	6.4682	25.7662	31.6453	18.9556	15.9919	10.9637	-
Mahalanobis distances (bold) and Procrustes distances (narrow).									
Abbreviations: AG, <i>A. grahami</i> ; CVO, <i>C. Vomitoria</i> ; CVI, <i>C. vicina</i> ; CM, <i>Ch. megacephala</i> ; CR, <i>Ch. rufifacies</i> ; HL, <i>He. ligurriens</i> ; LS, <i>L. sericata</i> ; PT, <i>Pr. terraenovae</i> ; TC, <i>T. calliphoroides</i>									

Table 4
Percentages of correct classification acquired from pairwise comparison of species with cross-validation test in MorphoJ.

		Group 2								
		AG	CVO	CVI	CM	CR	HL	LS	PT	TC
Group 1	AG	-	96.7	80	100	100	100	100	96.7	90
	CVO	96.7	-	93.3	100	100	100	100	100	93.5
	CVI	83.3	93.3	-	100	100	100	100	100	80
	CM	100	100	100	-	90	100	100	100	100
	CR	100	100	100	90	-	96.7	100	100	100
	HL	90	93.3	96.7	96.7	96.7	-	96.7	100	100
	LS	100	100	100	100	100	100	-	100	100
	PT	100	100	100	100	100	100	100	-	100
	TC	90	100	96.7	100	100	100	100	100	-
The values in bold are the percentages of the correct classification of group 1 obtained from group 1 compared with group 2; the values in narrow are the percentages of the correct classification of group 2 obtained from group 2 compared with group 1. Abbreviations: AG, <i>A. grahami</i> ; CVO, <i>C. Vomitoria</i> ; CVI, <i>C. vicina</i> ; CM, <i>Ch. megacephala</i> ; CR, <i>Ch. rufifacies</i> ; HL, <i>He. ligurriens</i> ; LS, <i>L. sericata</i> ; PT, <i>Pr. terraenovae</i> ; TC, <i>T. calliphoroides</i>										

Shape Variation

Canonical variate analysis (CVA) was used to analyze the most important shape characteristics between groups (genera or species) using MorphoJ software version 1.06 [40]. The CVA was used to magnify interspecific variation and minimize intraspecific variation. Mahalanobis distances were computed from DFA (discriminant function) as a statistical measure to evaluate the distance between groups, assessing the similarity between different populations. Permutation tests (10,000 replications) with Mahalanobis distances and Procrustes distances were used to test the statistical significance of pairwise differences between species and sexes. A cross-validated classification in discriminant function analysis (DFA) was used to evaluate the accuracy of species identification [40].

Allometry

Before the allometric analysis, a Procrustes ANOVA was used to test species and sex differences in allometry across the whole data set to evaluate the ability of allometries to identify species and sex (significant sex \times species interaction). Then, sex-specific multivariate regressions of shape on size were calculated for every species separately. The sex-dependent effect and species-dependent effect were analyzed with a permutation test of 10,000 rounds in MorphoJ software [40]. Besides, we also examined the results after removing allometry on species and sex discrimination. A cross-validation test of correctly classified specimens at the species level is conducted to compare the effects of species discrimination before and after removal of allometric effect.

Sexual Shape Dimorphism (sshd)

The DFA revealed no significant differences in wing shape between males and females. The percentage of correct classification of the males ranged from 60% (*He. ligurriens*) to 86.7% (*L. sericata*) while the percentage of correct classification of the females ranged from 46.7% (*Pr. terraenovae*) to 100% (*C. vomitoria*) (Table 8).

Table 8
Percentages of correct classification between sexes of nine blow fly species.

Species	No. of correctly classified specimens/total specimen No.	
	Males	Females
<i>A. grahami</i>	73.3(11/15)	80(12/15)
<i>C. vomitoria</i>	86.7(13/15)	100(15/15)
<i>C. vicina</i>	80(12/15)	80(12/15)
<i>Ch. megacephala</i>	73.3(11/15)	73.3(11/15)
<i>Ch. rufifacies</i>	66.7(10/15)	73.3((11/15)
<i>He. ligurriens</i>	60(9/15)	53.3(8/15)
<i>L. sericata</i>	86.7(13/15)	93.3((14/15)
<i>Pr. terraenovae</i>	80(12/15)	46.7(7/15)
<i>T. calliphoroides</i>	66.7(10/15)	73.3(11/15)

The percentages were obtained from cross-validation test using permutation test with 10,000 rounds in MorphoJ.

Phenetic Relationships Among Blow Flies

As shown in Fig. 4, the UPGMA dendrogram of nine species were divided into two distinct groups, with *Ch. rufifacies* and *Ch. Megacephala* always in a separate group, other species in the same subfamilies cluster into a cluster except *Pr. terraenovae*. The comparison of three pictures shows that the identification efficiency with male samples is better than that with female samples. In all three dendrogram, *C. vomitoria* was wrongly assigned to the branch of *He. ligurriens* and *L. sericata*.

Results

Phylogenetic analysis

The sequence of final alignment contained 111 variable sites, revealed a strong AT bias, with the average nucleotide compositions of A (30.0%), T (37.9%), C (17.4%), and G (14.7%), respectively. At the species level, the nine flesh fly species constituted their own monophyletic clusters with very strong supportive values. The NJ tree analyzed with the Tamura 3-parameter model showed that phylogenetic analyses of COI sequences yielded a tree of two distinct clades. The first clade contains two branches. The first branch consists of *C. vicina*, *Tr. calliphoroides*, *C. Vomitoria* and *A. grahami* while the second branch contains *He. ligurriens* and *L. sericata*. The second clade also includes two branches, one branch is *Pro. terraenovae*, the other branch containing *Ch. rufifacies* and *Ch. megacephala*. Although the samples come from different places, they can still form a cluster (Fig. 2) .

Intraspecific And Interspecific Divergence

The mean intraspecific variation ranged from 0.1–0.6% while distance analysis of intraspecific variation between each of the nine Calliphoridae species ranged from 4.3% (*C. vicina*/*T. calliphoroides*) to 13.6% (*Ch. rufifacies*/*L. sericata*) (Table 2). Analyses were conducted with the Kimura 2-parameter (K2P) model [36].

Table 2
Interspecific and intraspecific difference according to the analysis of their COI sequences.

	Centroid size (ANOVA)			Shape (ANOVA)						
	df	F	P	df	F	P				
Species	8	10.20	0.0018	256	25.75	<.0001				
Sex	1	4.69	0.0621	32	16.77	<.0001				
Sex × species	8	30.34	<.0001	256	3.71	<.0001				
Species	Intraspecific	Interspecific								
<i>A. grahami</i>	0.10	1	2	3	4	5	6	7	8	
<i>C. vicina</i>	0.30	6.90								
<i>C. vomitoria</i>	0.20	6.40	4.60							
<i>Ch. megacephala</i>	0.40	9.00	8.80	9.00						
<i>Ch. rufifacies</i>	0.60	11.10	11.30	11.10	7.00					
<i>He. ligurriens</i>	0.20	7.50	9.10	7.30	8.70	11.70				
<i>L. sericata</i>	0.20	8.30	7.50	6.70	9.50	13.60	5.70			
<i>Pr. terraenovae</i>	0.10	10.80	10.10	11.00	6.00	9.60	9.70	10.30		
<i>T. calliphoroides</i>	0.30	8.70	4.30	6.10	10.20	13.30	10.60	9.70	12.40	

Abbreviations: Ach: *Achoetandrus*; Ald: *Aldrichina*; Cal: *Calliphora*; Chr: *Chrysomya*; Hem: *Hemipyrellia*; Luc: *Lucilia*; Pro: *Protophormia*; Tri: *Triceratopyga*

Allometric Effects In Species And Sexes

The result of Procrustes ANOVA showed that allometries differed in species and sexes (significant sex × species interaction in Table 5), and sex has no significant influence on centroid size. The results of allometric analysis show that allometry accounted for 3.02% of the total shape variation, 2.49% of the female wings; for the male wings among nine species, allometry explained 2.16% of the total shape variation. The analysis of wing shape and size of each species revealed allometry in most species except for *C. vicina*, *L. sericata* and *Pr. terraenovae* (permutation test with 10,000 rounds in MorphoJ: $P > 0.05$). The wing shape variation was found to be significantly between sexes in *A. grahami*, *Ch. megacephala* and *Ch. rufifacies* (permutation test with 10,000 rounds in MorphoJ: $P < 0.0001$, $P < 0.01$, and $P < 0.05$) (Table 6). The percentage of correctly classified specimens at the species level in cross-validation test after removing the allometric effect is shown in Table 7.

Table 5
Results of Procrustes ANOVA on centroid size and shape.

Table 6
The predicted percentage in each blow fly species and between sexes of each species.

Species	predicted percentage within species	P-value	predicted percentage between sexes	P-value
<i>A.grahami</i>	8.1	0.0306	13.2	0.0293
<i>C.vomitorea</i>	15.4	0.0005	4.7	0.1381
<i>C.vicina</i>	2.4	0.6085	4.5	0.2023
<i>Ch.megacephala</i>	19.2	<.0001	6.2	0.0441
<i>Ch.rufifacies</i>	28.8	<.0001	6,3	0.0442
<i>He.ligurriens</i>	11.9	0.0044	4.2	0.2383
<i>L.sericata</i>	7.9	0.0539	5.3	0.1043
<i>Pr.terraenovae</i>	2.0	0.8132	4.1	0.2644
<i>T.calliphoroides</i>	11.3	<.0001	4.0	0.2951

Table 7
Percentages of correct classification acquired from pairwise comparison between species with cross-validation test*.

Group 2									
Group 1	AG	CVO	CVI	CM	CR	HL	LS	PT	TC
AG	-	93.3	83.3	100	100	90	100	100	96.7
CVO	93.3	-	93.3	100	96.7	93.3	100	100	100
CVI	80	96.7	-	100	100	96.7	100	100	100
CM	100	100	100	-	93.3	96.7	100	100	100
CR	100	100	100	90	-	100	100	100	100
HL	100	100	100	100	96.7	-	96.7	100	100
LS	100	100	100	100	100	100	-	100	100
PT	96.7	100	100	100	100	100	100	-	100
TC	86.7	96.7	80	100	100	100	100	100	-

*The cross-validation test is conducted in MorphoJ after removal of allometric growth using permutation test 10,000 rounds.

Discussion

In this study, we use only wing shape rather than size for analysis since previous studies have demonstrated that wing size cannot be used to distinguish species [27]. Compared with size, the shape of wings is less susceptible to environmental factors and has been proved to be a more stable feature [42, 43], which can provide very rich information for the phylogeny and evolution of organisms [44, 45]. The results suggested that wing morphometrics can be useful for identification genera and species of necrophagous Calliphoridae in China, which is consistent with previous studies in other countries [29, 38]. Except *He. ligurriens*, *Ch. megacephala* and *Ch. rufifacies*, overlapping classification was observed among some species, but the percentage of overall correct classification rate at the level of genus and species is high, ranging from 80–100% in genus and from 76.7–100% in species.

In morphological research, multivariate regression used to estimate allometry effects of different species is an important step in shape analysis since allometric growth can affect taxonomic research and sexual dimorphism [27]. Procrustes ANOVA has showed that allometries differed in species or sexes, and both species and sex have a significant effect on wing shape. The results in this study showed wing size explains part of the variation in wing shape among species, within a species, and between sexes in most species. This study confirmed that size correction by using residuals from the regression of shape on size was a necessary step in species identification based on shape [27]. Besides, the correct identification rate of species before and after the removal of allometric growth were compared in this study. We found that the identification accuracy after the removal of allometric growth was slightly lower than that keeping allometric growth. But allometry accounted for only 3.02% of the total shape variation, revealing that the allometry effects seem only a very small factor on the shape variation of species. However, to ensure the accuracy of identification, we suggest that, in the future laboratory research or practical application, the allometric growth should be removed first.

The differences between sexes were insignificant in all species ($P > 0.05$), which is slightly different from other studies. Significant difference was found between sexes of *C. vomitoria*, but not of *Ch. Megacephala* and *Pr. Terraenovae* collected in Europe [28] while differences between sexes existed in blow flies collected in Thailand, namely *Ch. Megacephala* and *He. Ligurriens* [27], indicating the morphology of Calliphoridae might differ in various locality of origin around the world. Therefore, the geographical populations can be identified according to morphometric analysis [38]. It is worth noting that to compare the effectiveness of morphometric analysis in male and female identification for certain species, the same number of males and females were used in each species to avoid the bias caused by different numbers. There were high accuracy rates of sex identification in some species like *C. vomitoria*, *C. vicina* and *L. sericata*, and the correct recognition rate of female *C. vomitoria* up to 100, indicating the wing shape can be used to identify sex for certain species, but more samples are needed to confirm this conclusion.

According to taxonomic criteria, these species belong to three subfamilies. The genus *Lucilia* and *Hemipyrellia* belong to *Luciliinae*; *Calliphora*, *Triceratopyga* and *Aldrichina* belong to *Calliphorinae*; *Protophormia* (*phormiini*), *Achoetandrus* and *Chrysomya* (*Chrysomyini*) belong to *Chrysomyinae*. The results of molecular analysis clearly divided into three clusters with *Luciliinae* and *Calliphorinae* in a large branch, *Chrysomyinae* in a small branch, and *phormiini* and *Chrysomyini* separated from others. In addition, phylogenetic trees show that the same species from different countries cluster in one branch. The interspecific distance of less than 1% indicated less difference between species collected from different countries or different places in China. Distance analysis of intraspecific variation between species showed that high percentage of intraspecific divergence between calliphorid species ranged from 4.3–13.6%, demonstrating that these species can be well distinguished by molecular methods. The

interspecific gaps between *C. vicina* and *C. vomitori* as well as *C. vicina* and *T. calliphoroides* are relatively low as they belong to the same subfamily, which proved the effectiveness of molecular species identification. However, as seen in Fig. 4, no matter female or male species were used in wing morphometrics, *C. vomitori* and *L. sericata* belonging to different subfamilies always formed in one branch. In contrast, through molecular methods, all species can be accurately identified to corresponding subfamilies, suggesting that the accuracy of molecular identification is higher than that of wing morphometrics in these species. Besides, the integrity of the wing must be ensured in wing morphometrics analysis.

The comparison results show that both molecular analysis and wing morphometrics can be used as important methods to distinguish species, but for certain species, wing morphometrics may give a wrong result. Meanwhile, molecular identification cannot distinguish geographical populations for some species [32]. Therefore, we recommend classical molecular identification for species identification to achieve better accuracy, and wing morphometrics can be used as a supplementary tool to identify geographical origin and sex. In the future, more species would be included to.

Conclusion

Our study results show that molecular analysis is more efficient for species identification, and wing morphometrics can be used as a complementary method combined with molecular methods or other methods to ensure the accuracy of identification. However, wing morphometrics has some advantages in identifying geographic population and sex of certain species. As a simpler and cheaper method, it is quite convenient for non-taxonomists to perform the identification after basic training [31, 35]. In the future research, larger sample size and other identification methods could be used to further test this conclusion and to achieve an optimal identification method set.

Abbreviations

PMI_{min}

Minimum postmortem interval

COI

Cytochrome c oxidase subunit I

K₂P

Kimura 2-parameter

NJ

Neighbor-joining

CVA

Canonical variate analysis

CV1

Canonical variate axis

CV2

Canonical variate axis

DFA

Discriminant function analysis

SSD

Sexual size dimorphism

UPGMA

Unweighted pair-group method with arithmetic averages

Declarations

Ethics approval and consent to participate

The study is approved by Ethics Committee of Central South University School of Basic Medical Sciences.

Consent for publication

All authors read and approved the final manuscript and give consent for publication.

Availability of data and materials

The data supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

All authors contributed and participated in the research and/or article preparation.

LXR and CTJ: preparing manuscript; SYJ: providing experimental advice, manuscript preparation and manuscript editing; RLP: analysis and editing manuscript; PPL: guiding data analysis; GYD: study design and providing advice; WY: study funding, data analysis, and manuscript editing; ZCQ: study design and preparing manuscript.

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Figures

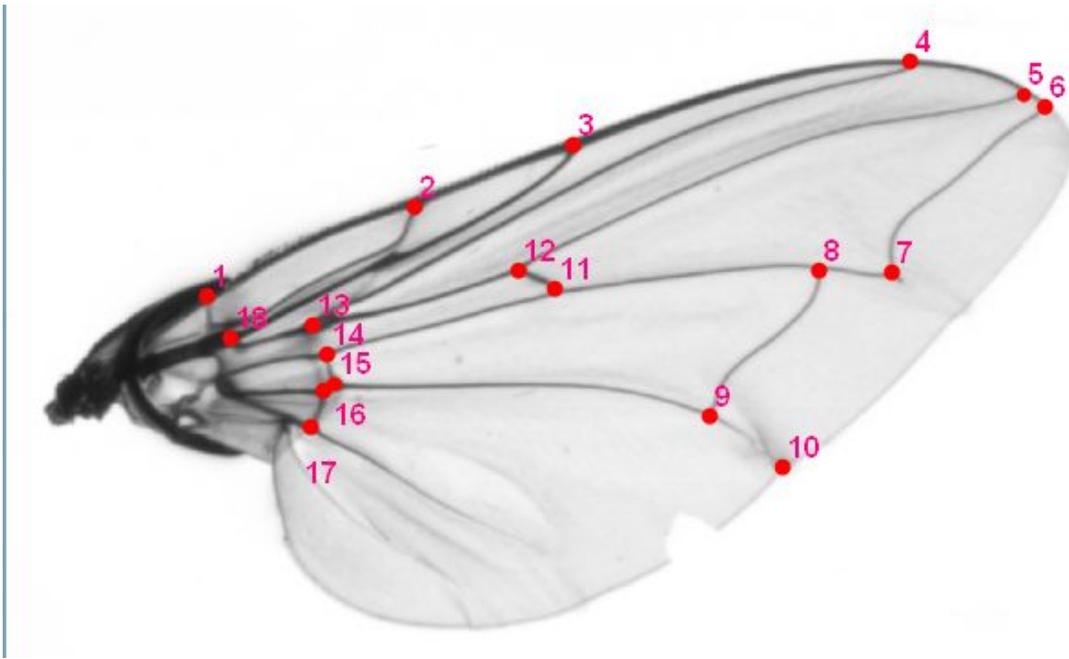


Figure 1

The right wing of female *T. calliphoroides* showing the reference of 18 plotted landmarks. The landmark selection is based on the method described by Hall et al.

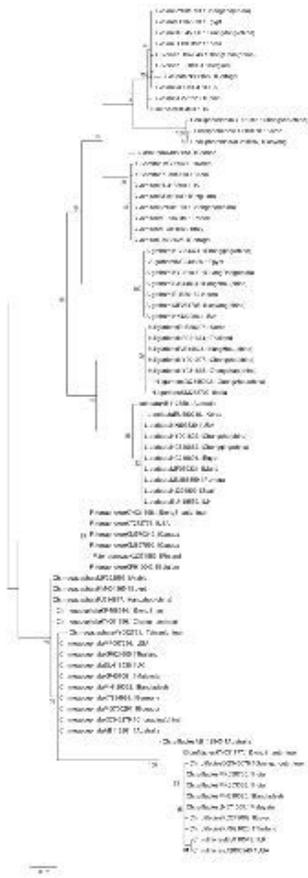


Figure 2

The neighbor-joining (NJ) tree constructed for 80 COI gene sequences from 9 species.

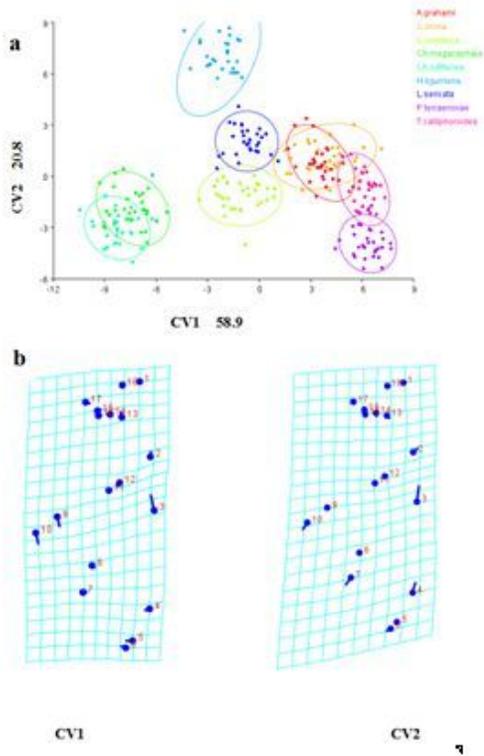


Figure 3

Scatter plot and transformation grids showing the variation in the shape of wings. (A) Scatter plot showing the variation in the shape of wings of 9 Calliphoridae species based on the first two canonical variate (CV1 and CV2) axes. (B) Transformation grids explain the shape changes from overall average along CV1 and CV2 axes.

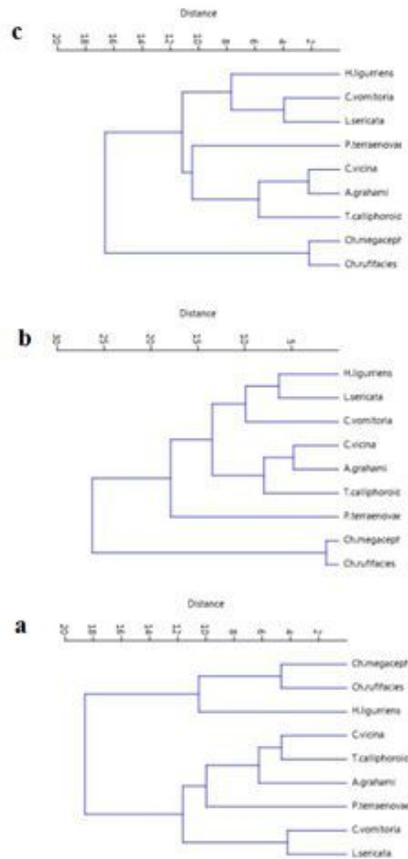


Figure 4

UPGMA dendrogram trees constructed using the specimens of nine species. (A) UPGMA similarity tree constructed using females from 9 species; (B) UPGMA similarity tree constructed using males from 9 species; (C) UPGMA similarity tree constructed using both male and female specimens.

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