Species-level identification of the blowfly *Chrysomya megacephala* and other Diptera in China by DNA barcoding

Journal:	<i>Genome</i>
Manuscript ID	gen-2015-0174.R2
Manuscript Type:	Article
Date Submitted by the Author:	12-Jul-2016
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Keyword:	blowfly, haplotype network, invasive species, Diptera, pest

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Species-level identification of the blowfly *Chrysomya megacephala* and other Diptera in China by
DNA barcoding

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

The blowfly *Chrysomya megacephala*, or oriental latrine fly, is the most common human-associated fly of the oriental and Australasian regions. *C. megacephala* is of particular interest for its use in forensic entomology and because it is a disease vector. The larvae are economically important as feed for livestock and in traditional Chinese medicine. Identification of adults is straightforward, but larvae and fragments of adults are difficult to identify. We collected *C. megacephala*, its allies *Chrysomya pinguis* and *Protophormia terraenovae*, as well as flies from 11 other species from 52 locations around China, then sequenced 658 base pairs of the COI barcode region from 645 flies of all 14 species, including 208 *C. megacephala*, as the basis of a COI barcode library for flies in China. While *C. megacephala* and its closest relative *C. pinguis* are closely related (mean K2P divergence of 0.022), these species are completely non-overlapping in their barcode divergences, thus demonstrating the utility of the COI barcode region for the identification of *C. megacephala*. We combined the 208 *C. megacephala* sequences from China with 98 others from public databases and show that worldwide COI barcode diversity is low, with 70% of all individuals belonging to one of three haplotypes that differ by one or two substitutions from each other, reflecting recent anthropogenic dispersal from its native range in Eurasia.

Keywords

Haplotype network, blowfly, invasive species, Diptera, pest

Introduction

The blowfly *Chrysomya megacephala* (Fabricius), or oriental latrine fly, is the most common human-associated fly of the oriental and Australasian regions (Wall and Shearer 1997). *C. megacephala* larvae develop in feces and decomposing flesh and consequently can be found at extremely high density (>95% of flies) under some environmental circumstances, such as locations near fish-processing activities (Wall et al. 2001). *C. megacephala* is native to Eurasia but through human action has spread around the world: by December 1975 it was reported from South America (Brazil) (Imbiriba et al. 1977) and later became established in New Zealand, Africa (Williams and Villet 2006), and then in North, South, and Central America via harbours and airports (Wells 1991; Williams and Villet 2006). It has a reported distribution across the whole of China except for arid high-elevation regions in Xinjiang, Qinghai, and Tibet (Xue and Zhao 1996).

C. megacephala is of particular importance to humans for a range of reasons: 1) it is considered as one of the most important fly species in the science of forensic entomology (Cai et al. 2005; Goff 2001; Shi et al. 2008; Wu and Hu 2012; Xue and Zhao 1996); 2) in traditional Chinese medicine *wuguchong*, the dried larva of *C. megacephala* is believed to have the curative effect of clearing stagnant heat-toxicity from the human body (Luo 1993); 3) live larvae are used in medicine in the form of “maggot therapy” (Taha et al. 2010); 4) it is an important source of animal feed protein (Sing et al. 2012); 5) it can cause myiasis (or fly strike) in sheep and occasionally in humans as it can invade open wounds (Bunchu et al. 2007); and 6) *C. megacephala* is also a disease vector and is known to lay eggs on human feces and subsequently transmit diseases such as bacterial gastroenteritis if it comes into contact with human food (Sukon et al. 2007). DNA barcoding has been successfully used for the molecular identification of a broad variety of insect taxa, including many Diptera (Nelson et al. 2007; Hernandez-Triana. 2015; Liao et al. Renaud et al. 2012; Rivera and Currie 2009; Schuehli et al. 2007), including *C. megacephala* and the closely related species *C. pinguis* (Nelson et al. 2012; Ramaraj et al. 2014; Salem et al. 2015). DNA barcoding, usually of a specific region in the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, generally relies on the observation that intraspecific COI variation is usually lower interspecific variation (Raupach et al. 2014). Consequently, comparative sequence analyses typically, but not always, reveal a “barcoding gap” (Meyer and Paulay 2005) on plots of pairwise

sequence differences and thereby allow molecular species-level identification of sequences generated from unidentified or unidentifiable samples, such as insect larvae or bloodstains (Hebert et al. 2003, 2004).

DNA barcoding has been criticized as a single-character typological approach that cannot replace systematic science and will not work for all clades (DeSalle et al. 2005; Ebach 2011; Klausnitzer 2010; Will et al. 2005). Nevertheless, it has become an important, useful, and increasingly used tool for species descriptions (Butcher et al. 2012; Hendrich and Balke 2011; Stoev et al. 2010; Tamura et al. 2013; Wesener 2012; Wesener et al. 2011) as well as various other biological disciplines Adamowicz 2015), including forensics (Ferri et al. 2009; Meiklejohn et al. 2011), pest biology (Engstrand et al. 2010), Inspection and Quarantine (Liao et al. 2014; Liu et al. 2014; Wei et al. 2014; Yue et al. 2013), and conservation biology (Neigel et al. 2007; Ward et al. 2008). Examples are the recommendation of barcoding for identification of flightless weevils in the genus *Trigonopterus* as a substitute for a traditional morphological key (Riedel et al. 2013); identifying the sources of food substitution or contamination (Cawthorn et al. 2012); identifying the presence of genetically modified organisms (Barcaccia et al. 2016); and identifying birds “minced” in jet engines (Wong and Hanner 2008; Grant 2007). In sum, DNA barcoding has proven both useful and reliable for species identification, particularly for degraded or partial specimens, for many taxonomic groups. This identification is only possible, though, if data from reliably identified specimens are available in public databases.

Adult *C. megacephala* are easily recognizable by experts, but less so for non-experts, while eggs, larvae, and fragments of adults, all of which may be encountered by pest control or public health workers, cannot be identified morphologically. A related question is whether *C. megacephala* exhibits any geographic structure that might allow assignment of place of origin to a sample of unknown provenance. Despite the ubiquity and economic importance of this species, *C. megacephala* barcode sequences, like those of many arthropods, are still poorly represented in public databases. In this study we collected *C. megacephala* as well as flies from 13 other species seven other genera, from 52 localities around China in order to confirm the utility of DNA for identification of the economically important *C. megacephala* and to establish a basic barcode library for *C. megacephala* and other related flies that co-occur with this species. There are 39 named species of the genus *Chrysomya* (<http://eol.org/pages/56219/overview> accessed 23 June

2016), of which three are common in China: *C. megacephala*, *C. pinguis*, and *C. phaonis*. *C.* commonly co-occurs with *C. megacephala* and is the closest relative of *C. megacephala* (Yang, et al 2014). We successfully collected individuals of *C. pinguis* and report the sequences here. We did not identify any *C. phaonis* specimens from our sampling and therefore cannot yet report *C. phaonis* barcodes, but adults of *C. phaonis* are morphologically distinct from *C. megacephala* (Yang, et al 2014) and it is very unlikely that *C. phaonis* samples would be confused for *C. megacephala*. We will report *C. phaonis* barcode sequences if samples become available in future. We also compared *C. megacephala* COI barcode sequences from our work with other publicly available *C. megacephala* sequences to assess whether variation within China is comparable to worldwide variation in this species.

Material and Methods

Sample collection

Adult flies were collected with a sweep net from 52 different localities in China during the summers of 2012 and 2013. As this was not an ecological study, we did not undertake random transects. Instead, to collect as many specimens as possible, we walked continually for up to two hours for a distance of roughly one kilometer, sweeping the nets frequently but also specifically targeting any flies we saw. Adults of all species collected were provisionally identified by morphology (Xue and Zhao 1996; Fan 1992). We also processed three individuals of *Musca domestica* that were intercepted in waste paper from California to Zhongshan (Guangdong China), three *C. megacephala* intercepted in waste paper from Manila, and six *C. megacephala* intercepted in waste paper from Lima. Specific permission was not required for collecting in these localities, and none of the species collected are endangered or protected. Identified specimens were verified and accessioned in the insect collection of Zhongshan Entry-Exit Inspection and Quarantine Bureau.

DNA barcoding

Genomic DNA extraction and PCR amplification

When available, we selected three specimens of each species at each collection locality for molecular analysis. A hind leg was removed from each specimen and placed in a 1.5 ml Eppendorf

tube with 95% ethanol. All instruments used to remove leg tissues were cleaned with 70% ethanol and flame sterilized between manipulation of each specimen. DNA was extracted from tissue following the standard protocols of the TIANamp Genomic DNA Kit (DP304, TIANGEN). The barcode region of COI was amplified using primer pair of LCO1490 and HCO2198 (Folmer et al. 1994).

Polymerase chain reactions were conducted in a 50 µl volume: 10× Taq polymerase buffer 5 µl, dNTP (2.5 mM each) 2 µl, primer (20 µM) 1 µl each, Taq polymerase (5 U/µl) 0.5 µl, DNA template 100 ng, add ddH₂O up to 50 µl. All PCR reagents were from TIANGEN (Beijing). Reaction conditions were 95°C 3 min; 95°C 45 s, 50°C 45 s, 72°C 1 min, 34 cycles; 72°C 10 min.

PCR product purification and sequencing

PCR products were purified and cloned as previously described: three colonies from each cloned PCR product were sequenced from both ends, and a consensus sequence from each clone was used for all analyses (Yue et al. 2014). Sequencing was successful for all individuals attempted.

Additional sequences

In addition to the sequences we generated from flies caught for this study, we also searched the Barcode of Life Data Systems (BOLD, Ratnasingham and Hebert 2007) public data portal for *Chrysomya megacephala* COI sequences and identified 98 sequences that included the barcode region, as generated for this study. The BOLD portal includes all current *C. megacephala* sequences in GenBank. BOLD accession numbers for these sequences are listed in supplemental information (Table S1). Only eight of these 98 sequences included latitude and longitude coordinates of the collection site. Eight of the sequences were incomplete at the 5' end: three were missing three bases and five were missing 13 bases. These were encoded as missing. All listed country of collection, with 37 listing a town, city, or other local place name. Since the majority of these sequences lacked precise geographic coordinates we used only country of collection to

Data analysis diagrams.

After removing primers, all sequences were 658 base pairs (bp) long and contained no deletions, or stop codons, and were translatable into the expected 219 residues of the COI gene. We used the MAFFT algorithm (<http://www.ebi.ac.uk/Tools/msa/mafft/>) to confirm the alignment.

Three separate datasets were extracted for intraspecific and interspecific analyses. For intraspecific analysis, we assembled one dataset with the 208 *C. megacephala* sequences, comprising 37 distinct haplotypes, from flies collected for this study and another of 306 *C. megacephala* sequences: the 208 from China plus the 98 downloaded from BOLD. This second dataset contained 53 unique haplotypes. The 208 “Chinese” flies included the three individuals from Manila and the six individuals from Lima that were intercepted at the port of Zhongshan, as these were collected in China. For interspecific analysis we assembled one dataset with sequences of 208 *C. megacephala*, 36 *C. pinguis*, and 13 *P. terraenovae* collected in China. For the intraspecific datasets, distances for each sequence pair were calculated as described below, assigned to a range interval, and the number of pairwise distances within each interval tallied and charted. For the two larger *C. megacephala*-only datasets, we also created network diagrams in order to examine geographic variation between *C. megacephala*. For the worldwide data set of 306 *C. megacephala* we assigned the nine individuals intercepted at the port of Zhongshan to their countries of origin (the Philippines and Peru) as representatives of haplotypes in those countries.

Mega version 6.06 was used for additional data analysis (Tamura et al. 2013). Mean frequencies (%) of each nucleotide and nucleotide pair (A+T and G+C) were calculated in MEGA to evaluate whether nucleotide frequencies were comparable to those typical of insects in general for this COI gene region (Renaud et al. 2012). We generated Kimura two-parameter (K2P) distances using the default parameters (transitions + transversions, gamma distribution) for the entire data set. Pairwise distance calculations in Mega ignore missing bases so some pairwise distances used slightly shorter total sequence lengths to calculate pairwise distances. We tested other distance models and note that our results were extremely robust and not sensitive to changes in the model used.

We used Microsoft Excel to tally the number of distance pairs in selected range intervals for a data set with *C. megacephala* and the two sympatric species whose barcode regions proved most similar to it: *C. pinguis* and *Protophormia terraenovae*. Our method allows changing the size of each interval, and again our results were robust over different interval ranges, merely changing the number of columns in each output chart. Network diagrams were constructed for *C. megacephala* sequences with PopART (Leigh and Bryant 2015).

To further explore the relationships between *C. megacephala*, *C. pinguis*, and *P. terraenovae*

we also undertook a phylogenetic analysis using a dataset in which each haplotype was as a single sequence. This dataset included 53 unique *C. megacephala*, 11 *C. pinguis*, and 8 *P. terraenovae* haplotypes, with a single *Achoetandrus rufifacies* sequence used as the outgroup. A maximum likelihood analysis was performed using Mega 6.06 using the T92+G model, which model testing within Mega 6.06 identified as the best model for these data using the criterion of lowest Bayesian information criterion (BIC) score. Additionally, we used Mega 6.06 to generate 1000 neighbor-joining bootstrap replicate distance trees, using maximum likelihood distance matrices generated using the same T92+G model.

Results

We sequenced the COI barcode region of 645 fly specimens from 14 species in three families, including two *Chrysomya* species (*C. megacephala*, *C. pinguis*) and six other calliphorids. The mean nucleotide content of the COI sequences was A (30.0%), T (38.4%), G (15.7%), and C (15.9%). A + T (68.4%) was in higher proportion than G + C (31.6%) and was comparable to those typical of insects in general for this COI gene region and for other dipteran mitochondrial sequences (Renaud et al. 2012; Rivera and Currie 2009; Schuehli et al. 2007). Collection locations are mapped in Fig. 1.

The focus of this project was barcoding *C. megacephala* and its close relatives; hence, 208 of 645 sequenced flies were *C. megacephala*, and 83 others were *C. pinguis*, *Achoetandrus rufifacies*, and *Protophormia terraenovae*, all from the Calliphoridae. *C. pinguis* is believed to be the closest relative of *C. megacephala* in China (Yang et al. 2014), and the evidence from the work reported here supports this conclusion, but we note that COI barcode sequences are not yet available for the less common *C. phaonis*. *A. rufifacies*, *C. megacephala*, and *C. pinguis* are all classified within the subfamily Chrysomyinae, while *P. terraenovae* is classified in a separate subfamily, the Phormiinae, but mean K2P distances between *C. megacephala* and *A. rufifacies* were 0.07, with a range of 0.065 to 0.08, about 15% greater than the distances between *C. megacephala* and *P. terraenovae*. Therefore, our subsequent distance analyses use *P. terraenovae* as a third species rather than *A. rufifacies*.

Sequences from the other 11 species are reported here and have been submitted to GenBank, are not otherwise analyzed due to relatively small numbers of sequences and to their taxonomic

distance from *C. megacephala*, which is the focus of this paper. Table 1 lists all species and the number of individuals sequenced. Collection information and GenBank accession numbers for all sequenced specimens are summarized in supplemental Table S2.

Interspecific variation in *Chrysomya megacephala*, *Chrysomya pinguis*, and *Protophormia terraenovae* in China

A primary goal of this work was to determine whether fly larvae and other difficult-to-identify samples—such as fragments of adult bodies—can be identified using mitochondrial COI barcode sequences. We tested the practical utility of barcoding for identifying specimens to the species level using pairwise comparisons between all individuals of *C. megacephala* and each of the two other sympatric species with the most similar COI barcoding regions: *C. pinguis* and *P. terraenovae* (Fig. 2). These results show a mean intraspecific Kimura two-parameter (K2P) distance between individuals of *C. megacephala* of 0.0028 and mean interspecific K2P distance between individuals of *C. megacephala* and *C. pinguis* of 0.022. Significantly, there is no overlap between the intra- and inter-specific distributions, with a minimum interspecific K2P distance of 0.016 between any two individuals of different species and a maximum intraspecific K2P distance of 0.011 among individuals of *C. megacephala*. Our analysis of interspecific differences is of course based on a modest number of sequenced individuals: 13 *P. terraenovae*, 36 *C. pinguis*, and 208 *C. megacephala* and should be confirmed by collection and sequencing of additional individuals. Nevertheless, the results are strong enough that we are confident the COI barcoding region is useful for differentiating biological material between the two *Chrysomya* congeners in China. A phylogenetic analysis (Figure S1) separates *C. megacephala*, *C. pinguis*, and *P. terraenovae* into three distinct clades with 99 percent bootstrap support, further confirming the utility of the COI barcode region for distinguishing these three species. Barcode sequences from the other 11 species that we collected are significantly different, and readily differentiated, from those of *C. megacephala* and are not further discussed.

Intraspecific variation in *Chrysomya megacephala*

The analysis above demonstrated that COI barcode sequences differentiate *C. megacephala* from its close relative in China. To understand whether *C. megacephala* exhibits any geographic structure that might allow assignment of place of origin to a sample of unknown provenance, we assembled and analyzed two datasets, one with the 208 *C. megacephala* sequences including 37

haplotypes from individuals captured for this study and a second expanded dataset with those 208 sequences as well as 98 more from the BOLD database to examine this question.

In the network diagram six of the haplotypes were represented by 5 or more specimens, with one including 97 specimens—almost half of the entire dataset, and two others with 37 and 11 identical specimens (Fig. 3a). These three haplotypes, differing by only one or two base pairs, include 70% of the entire dataset. Five haplotypes were represented by three individuals, one was observed from two individuals, and 24 were singletons. The network diagram shows that most of the sequences are just a few mutation steps away from one of the three large haplotype groups. There is some possible geographic structure, as individuals from Hainan and the southwest (Sichuan and Yunnan) do not appear in the 37-member haplogroup or its near neighbours. However, there are no diagnostic haplotypes that would clearly identify an individual as belonging to a certain geographic area, and the sample sizes from Hainan (11 individuals) and the southwest (9 individuals) are too low to confirm this observation for these regions. We cannot conclude at present that COI barcodes are useful for identifying the geographic origin of *C. megacephala* within China.

Fig. 3b combines the 208 sequences from flies sequenced for this study with 98 publicly available sequences from BOLD that originated in nine different countries. The pattern of diversity in this network is identical to the pattern shown in Fig. 3a, with the same three large closely related haplotype groups. The maximum path length through the network is 13 steps. Flies from Malaysia and Egypt are represented in the two largest haplotype groups, whereas flies from other geographic regions are represented only in the largest group, with the exception of a single individual from Australia on its own branch two steps from the largest haplogroup. As with flies originating in China, there are no clearly identifiable markers for geographic origin in this dataset. Given the relatively small sample sizes (excepting Malaysia_Singapore) this result is not surprising, particularly since *C. megacephala* is an introduced species in Egypt, Australia, and the Americas and may have reduced mitochondrial diversity due to founder effects. Nevertheless, the overall similarity of the networks in Fig. 3a and Fig. 3b suggests that we have in these data captured most of the worldwide diversity within the COI barcode region for *C. megacephala*.

Discussion

C. megacephala has been reported throughout China, from the plains of the eastern coastal areas to the Inner Mongolian plateau and into the hills and mountains bordering the Tibetan plateau: that is, all provinces except for Xinjiang, Qinghai, and Tibet (Xue and Zhao 1996). Lack of records from these three provinces may be due to inadequate sampling during periods when this fly is active, as was found to be true in South Africa (Williams and Villet 2006). Our field collection confirmed the distribution of *C. megacephala* throughout eastern China, and four of us (QY, DL, KW, JC) also spent two weeks in Tibet (28th of June to 9th of July of 2013) searching for flies from Lhasa to Nyingchi (including Nyingchi city, Motuo and Paizhen), with no success (Fig. 1). There are also records for *C. megacephala* in Inner Mongolia, and we did capture flies in Hohhot, the capital city of Inner Mongolia, but we did not find any individuals either in Erenhot or in Manzhouli, at the international borders with Mongolia and Russia. Nor did we find any individuals in Mohe, Heilongjiang Province, at the most northern international border with Russia. Our results confirm previous work (Xue and Zhao 1996) that *C. megacephala* does not occur in the far western, northwestern, or northern border regions of China. These are regions of extreme cold, little rainfall, and, in the far west, of high elevation, suggesting that this species cannot survive low temperatures, arid conditions, or both.

The primary goal of this study was to determine whether *C. megacephala*, which is an economically and forensically important fly, can be unambiguously identified in China using the COI barcoding region. Our results show that both *C. megacephala* and *C. pinguis*, its closest relative in China, are easily distinguished using the COI barcode region, with no overlap between the intra- and inter- specific distributions (Fig 2).

We were interested in whether the three *C. megacephala* individuals from Manila and six from Lima that were intercepted at the port of Zhongshan were identifiably different from other flies in China, but all nine belonged to the most common COI haplotype. This is a clear indication of the cosmopolitan distribution and recent anthropogenic dispersal (Imbiriba et al. 1997; Wall et al. 2001; Wells 1991).

The two other calliphorids closest to the two *Chrysomya* spp. among the species sampled here, *Achoetandrus rufifacies* and *Protophormia terraenovae*, are also easily identified using the barcoding region, with well over 5% divergence between these and either *Chrysomya* spp. Interestingly, *A. rufifacies* is currently classified in the same subfamily, the Chrysomyinae, as

Chrysomya spp., but in fact the COI sequences of *P. terraenovae*, currently assigned in the subfamily Phormiinae, were more similar to those of the two species of *Chrysomya* than were those of *A. rufifacies*. Given the small number of individuals of *A. rufifacies* and *P. terraenovae*, and the short length of the COI barcoding region, this result is not definitive, but does suggest that additional work on the phylogeny of the Chrysomyinae might be warranted.

Comparison of the network diagram showing *C. megacephala* collected in China (Fig. 3a) to that showing worldwide *C. megacephala* (Fig. 3b) suggests that variation in the barcode region we observed within China includes most of the variation seen worldwide for this species, and we hypothesize that additional sequencing for this species will not expand the network significantly. Again, though, sample sizes and geographic sampling were low outside of China, so additional work is needed to reach a definitive conclusion. Nevertheless, given the very high frequency of three very closely related haplotypes, it is clear that most samples from *C. megacephala* collected from anywhere in its worldwide range should be unambiguously identifiable using the tools available on the Barcode of Life Data Systems portal or even with a standard BLAST search. In fact, our results suggest that a majority of all collected individuals would have one of the three most common sequences.

Fieldwork is time consuming and expensive, and collecting by sweep net is imprecise, so our collections, as described above, included hundreds of individuals from a number of other species. The number of individuals from each of these other species was too low for the robust analysis that we have presented for *C. megacephala*, but these samples are, nevertheless, important additions to the corpus of publicly available barcodes from Chinese insects. Barcodes were generated from a single leg of each individual and released publicly (Table S2); the rest of each fly has been deposited as voucher specimens into the collection of the Zhongshan Entry-Exit Inspection and Quarantine Bureau, where they are available for additional study. Such collections play a vital role in providing information on the location and spread of living organisms, and, like sequence databases, become more useful as more samples are added, regardless of how familiar or common the species might appear to be (Williams and Villet 2006).

Acknowledgments

This work was financially supported by National Science and Technology support program

“2012BAK11B05”, AQSIQ support program “2015IK067, 2015IK069” Guangdong Province support program “2015A050502009”.

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Table 1. Species and number of individuals for which mitochondrial COI barcodes were sequenced.

Family	Sub-family	Genus	Specific epithet	No. of individuals
Calliphoridae	Chrysomyinae	<i>Chrysomya</i>	<i>megacephala</i>	208
			<i>pinguis</i>	36
		<i>Achoetandrus</i>	<i>rufifacies</i>	34
	Phormiinae	<i>Protophormia</i>	<i>terraenovae</i>	13
	Calliphorinae	<i>Lucilia</i>	<i>illustris</i>	10
			<i>cuprina</i>	36
			<i>sericata</i>	47
			<i>Hemipyrellia</i>	41
			<i>ligurriens</i>	41
Muscidae	Muscinae	<i>Musca</i>	<i>domestica</i>	50
			<i>sorbens</i>	33
	Coenosiinae	<i>Graphomya</i>	<i>rufitibia</i>	40
Sarcophagidae	Sarcophaginae	<i>Sarcophaga</i>	<i>albiceps</i>	33
			<i>brevicornis</i>	32
			<i>peregrina</i>	32

Figure captions

Fig. 1. Collecting localities of *Chrysomya megacephala* and other Diptera in China. The 42 solid circles are the localities where *C. megacephala* were found, the ten empty circles are the localities, at high altitude on the Tibetan Plateau, in arid areas near the Mongolian border, and in low temperature areas near the Russian border, where we searched for but did not find *C. megacephala*. Map data ©2015 Google.

Fig. 2. Kimura two-parameter (K2P) pairwise sequence distances of the 658 bp COI barcoding region between 208 individuals of *Chrysomya megacephala*, 36 *C. pinguis*, and 13 *Protophormia terraenovae* (Cmeg, Cpin, and Pter) collected in China. These included nine individuals intercepted at the port of Zhongshan: six from Lima, Peru and three from Manila, the Philippines as those individuals were collected in China. The maximum K2P distance between two *C. megacephala* is 0.011, while the minimum between *C. megacephala* and *C. pinguis* is 0.016. The mean Cmeg/Cmeg distance is 0.0028, while the mean Cmeg/Cpin distance is 7.9-fold greater at 0.0220. It is clear that sequences of the COI barcode region are sufficient to distinguish biological material from these two species. Distances to *P. terraenovae* are considerably greater, as were pairwise distances for other collected flies (data not shown).

Fig. 3. Minimum spanning network diagrams for *Chrysomya megacephala* for the 658 bp COI barcode region. Minimum spanning networks were created using PopART, with epsilon of 0. Both data sets are robust in that nearly identical topologies are produced regardless of which network algorithm is used. (a) Network showing 208 sequences collected in China. Collection sites were assigned to one of seven regions within China and encoded in a nexus traits block. The nine sequences intercepted at the port of Zhongshan (six from Lima, three from Manila), all sharing the same most common haplotype, were assigned to the Fujian-Guangzhou-Guangxi region where they were collected. Numbers next to regional name indicate number of sequences from that region. Each circle represents one or more identical sequences, with circle size proportional to the number of sequences. Numbers beside larger circles indicate number of sequences within that group. Branch lengths and angles are arbitrary: each hash line across a branch indicates a single mutation. The maximum path length across the network is 11 mutations. Colors indicate geographic origin of the sequences within each group. Sequences from northern and central China (the top five regions in the key) occur throughout the network. However, individuals from Hainan occur only within, and branching from, the 97-sequence group, while individuals from Sichuan and Yunnan occur only within the 97-sequence group and branching from the 11-sequence group at the top center. (b) Network showing 306 *C. megacephala* sequences; 208 as in (a) plus 98 additional sequences from the Barcode of Life Data Systems (Ratnasingham and Hebert 2007). These sequences included six additional individuals from China, but for this network we assigned the six sequences originating in Manila and the six originating in Lima, but intercepted at Zhongshan, to their country of origin as representatives of genotypes that are present in the Philippines and Peru. Again, all nine share the most common haplotype. As in (a) individuals from all countries are present in the largest haplogroup. The second largest haplogroup is limited to flies

from China, Malaysia, and Egypt. Most of the variation in the data is within the flies we collected in China, as expected given the larger relative sample size and the widespread collection locations.

Draft

Fig. 1. Collecting localities of *Chrysomya megacephala* and other Diptera in China. The 42 solid circles are the localities where *C. megacephala* were found, the ten empty circles are the localities, at high altitude on the Tibetan Plateau, in arid areas near the Mongolian border, and in low temperature areas near the Russian border, where we searched for but did not find *C. megacephala*. Map data ©2015 Google.

128x91mm (300 x 300 DPI)

Fig. 2. Kimura two-parameter (K2P) pairwise sequence distances of the 658 bp COI barcoding region between 208 individuals of *Chrysomya megacephala*, 36 *C. pinguis*, and 13 *Protophormia terraenovae* (Cmeg, Cpin, and Pter) collected in China. These included nine individuals intercepted at the port of Zhongshan: six from Lima, Peru and three from Manila, the Philippines. The maximum K2P distance between two *C. megacephala* is 0.011, while the minimum between *C. megacephala* and *C. pinguis* is 0.016. The mean Cmeg/Cmeg distance is 0.0028, while the mean Cmeg/Cpin distance is 7.9-fold greater at 0.0220. It is clear that sequences of the COI barcode region are sufficient to distinguish biological material from these two species. Distances to *P. terraenovae* are considerably greater, as were pairwise distances for other collected flies (data not shown).

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209x244mm (300 x 300 DPI)

Draft

Table S1. Publicly available *Chrysomya megacephala* COI barcode sequences downloaded from the Barcode of Life Data Systems (BOLD) public data portal. BOLD accession number, GenBank accession number, and country of collection are listed.

BOLD accession	GenBank accession	Country
GBDP14020-13	NC_019633	Australia
GBDP14083-13	JX913739	Australia
GBDP14084-13	JX913738	Australia
GBDP3477-07	DQ647353	Australia
GBDP3478-07	DQ647352	Australia
GBDP3479-07	DQ647351	Australia
GBDP3480-07	DQ647350	Australia
GBDP15509-14	KJ195707	Brazil
GBDP15510-14	KJ195708	Brazil
GBDP15512-14	KJ195714	Brazil
GBDP0975-06	AY092761	China
GBDP14428-13	KF037970	China
GBDP14429-13	KF037969	China
GBDP9050-10	FJ614818	China
GBDP9051-10	FJ614817	China
GBDP9052-10	FJ614816	China
GBDP15505-14	KC249673	Egypt
GBDP15506-14	KC249674	Egypt
GBDP15507-14	KC249675	Egypt
GBDP15508-14	KC249676	Egypt
GBDP0583-06	AF295551	India
GBDP15222-14	AB907185	India
GBDP15230-14	AB910389	India
GBDP15231-14	AB910390	India
GBDP2900-07	AJ426041	India
SPLID013-13		India
SPLID033-14		India
GBDP13116-13	KC855286	Malaysia
GBDP13130-13	KC855272	Malaysia
GBDP13131-13	KC855271	Malaysia
GBDP13132-13	KC855270	Malaysia
GBDP15270-14	KF562106	Malaysia

GBDP15513-14	KJ496781	Malaysia
GBDP15514-14	KJ496782	Malaysia
GBDP15515-14	KJ496783	Malaysia
GBDP15516-14	KJ496784	Malaysia
GBDP15517-14	KJ496785	Malaysia
GBDP1869-06	AY909052	Malaysia
GBDP1870-06	AY909053	Malaysia
GBMIN21190-13	JX187374	Malaysia
GBMIN21191-13	JX187372	Malaysia
GBMIN21192-13	JX187370	Malaysia
GBMIN21193-13	JX187368	Malaysia
GBMIN21319-13	JX187373	Malaysia
GBMIN21320-13	JX187371	Malaysia
GBMIN21321-13	JX187369	Malaysia
GBMIN22942-13	JX027581	Malaysia
GBMIN22943-13	JX027579	Malaysia
GBMIN22944-13	JX027577	Malaysia
GBMIN22945-13	JX027575	Malaysia
GBMIN22946-13	JX027573	Malaysia
GBMIN22947-13	JX027571	Malaysia
GBMIN22948-13	JX027569	Malaysia
GBMIN22949-13	JX027567	Malaysia
GBMIN22950-13	JX027565	Malaysia
GBMIN22951-13	JX027563	Malaysia
GBMIN22952-13	JX027561	Malaysia
GBMIN22953-13	JX027559	Malaysia
GBMIN22954-13	JX027557	Malaysia
GBMIN22955-13	JX027555	Malaysia
GBMIN22956-13	JX027553	Malaysia
GBMIN22957-13	JX027551	Malaysia
GBMIN22958-13	JX027549	Malaysia
GBMIN22961-13	JX027580	Malaysia
GBMIN22962-13	JX027578	Malaysia
GBMIN22963-13	JX027576	Malaysia
GBMIN22964-13	JX027574	Malaysia
GBMIN22965-13	JX027572	Malaysia
GBMIN22966-13	JX027570	Malaysia
GBMIN22967-13	JX027568	Malaysia
GBMIN22968-13	JX027566	Malaysia
GBMIN22969-13	JX027564	Malaysia
GBMIN22970-13	JX027562	Malaysia
GBMIN22971-13	JX027560	Malaysia
GBMIN22972-13	JX027558	Malaysia

GBMIN22973-13	JX027556	Malaysia
GBMIN22974-13	JX027554	Malaysia
GBMIN22975-13	JX027552	Malaysia
GBMIN22976-13	JX027550	Malaysia
GBMIN22977-13	JX027548	Malaysia
GBMIN30954-13	JN229003	Malaysia
GBMIN30956-13	JN228999	Malaysia
GBMIN30958-13	JN228995	Malaysia
GBMIN30961-13	JN229000	Malaysia
GBMIN30963-13	JN228996	Malaysia
GBMIN30964-13	JN228994	Malaysia
GBMIN32602-13	JN571566	Malaysia
GBMIN32603-13	JN571564	Malaysia
GBMIN32604-13	JN571562	Malaysia
GBMIN32607-13	JN571556	Malaysia
GBMIN32608-13	JN571554	Malaysia
GBMIN32614-13	JN571561	Malaysia
GBMIN32617-13	JN571555	Malaysia
GBMIN32618-13	JN571553	Malaysia
DIRTT059-11	KC617813	United States
DIRTT060-11	KC617814	United States
DIRTT061-11	KC617812	United States
GBMIN18761-13	JQ246662	United States

Table S2Collection localities, geographic information, and COI barcode region GenBank accession numbers for each individual fly.

Species	Collection Locality	Province	GPS Coordinates		Internal specimen ID	GenBank Accession No.	Collector	Collection Date (yyyymmdd)
			Long.(E)	Lat. (N)				
<i>Chrysomya megacephala</i>	Chaohu	Anhui	117.872	31.642	28Ad-1	KJ129130	Wang XD	20121002
					28Ad-2	KJ129131		
					28Ad-3	KJ129132		
	Luan		116.333	31.393	28P-1	KJ129098	Wang XD	20120903
					28P-2	KJ129099		
					28P-3	KJ129100		
	Chongqing	Chongqing	106.429	29.821	28T-1	KJ129110	Liu DX	20130725
					28T-2	KJ129111		
					28T-3	KJ129112		
	Fuzhou	Fujian	119.300	26.150	28I-1	KJ129080	Wei XY	20120928
					28I-2	KJ129081		
					28I-3	KJ129082		
					28I-4	KJ129083		
	Xiamen		118.176	24.518	28G-1	KJ129073	Wang XD	20120918
					28G-2	KJ129074		
					28G-3	KJ129075		
					28G-4	KJ129076		
	Wuyishan		118.004	27.705	28H-1	KJ129077	Wei XY	20120923
					28H-2	KJ129078		
					28H-3	KJ129079		
	Huizhou	Guangdong	114.509	23.177	28D-1	KJ129062	Yue QY	20120519
					28D-2	KJ129063		
					28D-3	KJ129064		
					28D-4	KJ129065		
	Meizhou		116.089	24.271	28Q-1	KJ129101	Qiu DY	20121004
					28Q-2	KJ129102		
					28Q-3	KJ129103		
	Shantou		116.712	23.403	28B-1	KJ129054	Yue QY	20120518
					28B-2	KJ129055		
					28B-3	KJ129056		
					28B-4	KJ129057		
	Yunfu		112.059	22.912	28C-1	KJ129058	Liu DX	20121001
					28C-2	KJ129059		
					28C-3	KJ129060		
					28C-4	KJ129061		
	Zhongshan		113.423	22.517	28A-1	KJ129053	Huang YW	20110315
					28A-2	KJ145953		
					26A	KP310058		20110823
	Fangchenggang	Guangxi	108.055	21.892	28Aa-1	KJ129133	Wang XD	20130407
					28Aa-2	KJ129134		
	Chongzuo		106.961	22.468	26O-1	KP408518	Wu KL	20140505
					26O-2	KP408519		
					26O-3	KP408520		
					26P-1	KP408521		
					26P-2	KP408522		
					26P-3	KP408523		
	Haikou	Hainan	110.316	20.034	28M-1	KJ129090	Wang XD	20121126
					28M-2	KJ129091		
					28M-3	KJ129092		
	Ledong		108.863	18.740	28N-1	KJ129093	Wang XD	20130429
					28N-2	KJ129094		
	Sanya		109.508	18.256	28K-1	KJ129085	Wang XD	20121120
					28K-2	KJ145954		
	Wuzhishan		109.671	18.880	28L-1	KJ129086	Wang XD	20121124
					28L-2	KJ129087		
					28L-3	KJ129088		
					28L-4	KJ129089		
	Shijiazhuang	Hebei	114.411	38.070	28Ab-1	KJ129125	Liu DX	20120822
					28Ab-2	KJ145956		

	Qinhuangdao		119.485	39.835	28Ab-3	KJ129126	Wang XD	20120918
					28F-1	KJ129069		
					28F-2	KJ129070		
					28F-3	KJ129071		
	Zhengzhou	Henan	113.673	34.900	28W-1	KJ129119	Qiu XY	20130812
					28W-2	KJ129120		
					28W-3	KJ129121		
	Yueyang	Hunan	113.092	29.373	28Z-1	KJ129122	Chen J	20130821
					28Z-2	KJ129123		
					28Z-3	KJ129124		
	Xiangyang	Hubei	112.178	32.045	28Y-1	KJ129141	Chen J	20130819
					28Y-2	KJ129142		
					28Y-3	KJ129143		
					28Y-4	KJ129144		
					28Y-5	KJ129145		
	Xiaogan		114.120	31.556	28J-1	KJ129084	Wei XY	20120930
					28J-2	KJ145955		
	Hohhot	Inner Mongolia	111.653	40.752	28U-1	KJ129113	Hu J	20130804
					28U-2	KJ129114		
					28U-3	KJ129115		
	Nanjing	Jiangsu	118.746	32.087	28O-1	KJ129095	Liu DX	20120831
					28O-2	KJ129096		
					28O-3	KJ129097		
	Jinan	Shandong	117.025	36.675	28Ac-1	KJ129127	Wang XD	20120823
					28Ac-2	KJ129128		
					28Ac-3	KJ129129		
	Weinan	Shaanxi	109.430	34.517	28X-1	KJ129136	Chen J	20130817
					28X-2	KJ129137		
					28X-3	KJ129138		
					28X-4	KJ129139		
					28X-5	KJ129140		
	Datong	Shanxi	113.190	40.106	28V-1	KJ129116	Chen J	20130810
					28V-2	KJ129117		
					28V-3	KJ129118		
	Emeishan	Sichuan	103.493	29.591	28S-1	KJ129107	Wu KL	20130722
					28S-2	KJ129108		
					28S-3	KJ129109		
	Panzhihua		101.636	26.711	28R-1	KJ129104	Wu KL	20130716
					28R-2	KJ129105		
					28R-3	KJ129106		
	Tianjin	Tianjin	117.488	40.022	28E-1	KJ129066	Wang XD	20120816
					28E-2	KJ129067		
					28E-3	KJ129068		
	Botanical Garden,Dalian	Liaoning	121.659	38.909	26E-1	KP408488	Liu DX	20140828
					26E-2	KP408489		
					26E-3	KP408490		
					26F-1	KP408491		
					26F-2	KP408492		
					26F-3	KP408493		
					26G-1	KP408494		
					26G-2	KP408495		
					26G-3	KP408496		
					26H-1	KP408497		
					26H-2	KP408498		
					26H-3	KP408499		
					26I-1	KP408500		
					26I-2	KP408501		
					26I-3	KP408502		
					26J-1	KP408503		
					26J-2	KP408504		
					26J-3	KP408505		
	Fujiashuang Park, Dalian		121.623	38.865	26K-1	KP408506	Chen J	20140829
					26K-2	KP408507		
					26K-3	KP408508		

					26L-1	KP408509		
					26L-2	KP408510		
					26L-3	KP408511		
					26M-1	KP408512		
					26M-2	KP408513		
					26M-3	KP408514		
					26N-1	KP408515		
					26N-2	KP408516		
					26N-3	KP408517		
	Xixi National Wetland Park, Hangzhou	Zhejiang	120.067	30.269	26Q-1	KP408524	Liao JL	20140825
					26Q-2	KP408525		
					26Q-3	KP408526		
	Precious Stone Hill, Hangzhou		120.144	30.261	26R-1	KP408527		20140826
					26R-2	KP408528		
					26R-3	KP408529		
	Tianmushan, Hangzhou		119.429	30.348	26S-1	KP408530		20140822
					26S-2	KP408531		
	Longshan park, Jinhua		119.597	28.625	26U-1	KP408535	Wei XY	20140829
					26U-2	KP408536		
					26U-3	KP408537		
					26V-1	KP408538		20140827
					26V-2	KP408539		
					26V-3	KP408540		
	Moon garden, Jinhua		119.668	29.086	26W-1	KP408541		20140831
					26W-2	KP408542		
					26W-3	KP408543		
	Huangbinhong park, Jinhua		119.652	29.096	26X-1	KP408544		20140901
					26X-2	KP408545		
					26X-3	KP408546		
	Jiujiang	Jiangxi	116.003	29.711	26T-1	KP408532	Wu KL	20140907
					26T-2	KP408533		
					26T-3	KP408534		
	Harbin	Heilongjiang	127.155	45.567	26Z-1	KP408547	Chen J	20140806
					26Z-2	KP408548		
					26Z-3	KP408549		
	Longfeng marsh Park, Daqing		125.096	46.515	19C-1	KP408460	Liu DX	20140818
					19C-2	KP408461		
					19C-3	KP408462		
	Times square, Daqing		125.101	46.582	19D-1	KP408463		20140817
					19D-2	KP408464		
					19D-3	KP408465		
	People's Park, Mudanjiang		129.627	44.588	19E-1	KP408466	Chen J	20140822
					19E-2	KP408467		
					19E-3	KP408468		
	Jiangdong, Mudanjiang		129.119	44.113	19F-1	KP408469		20140820
					19F-2	KP408470		
	Heihe		126.170	48.652	19G-1	KP408471	Yue QY	20140816
					19G-2	KP408472		
					19G-3	KP408473		
					19H-1	KP408474		
					19H-2	KP408475		
					19H-3	KP408476		
	Longsha Park, Qiqihar		123.944	47.344	19K-1	KP408483		20140813
					19K-2	KP408484		
	Peace Square, Qiqihar		123.919	47.360	19L-1	KP408485		20140812
					19L-2	KP408486		
					19L-3	KP408487		
	Jilin	Jilin	126.702	43.721	19A-1	KP408454	Chen J	20140826
					19A-2	KP408455		
					19A-3	KP408456		
					19B-1	KP408457		
					19B-2	KP408458		
					19B-3	KP408459		
	Hailan riverside, Yanji		129.427	42.787	19I-1	KP408477	Liu DX	20140823
					19I-2	KP408478		
					19I-3	KP408479		

	Hailan Lake, Yanji		129.633	42.913	19J-1	KP408480		20140824	
					19J-2	KP408481			
					19J-3	KP408482			
	-----	Manila, Philippines	-----	-----	26B-1	KP310059	Nie WZ	20140308	
					26B-2	KP310060			
					26B-3	KP310061			
					26C-1	KP310062			
					26C-2	KP310063			
					26C-3	KP310064			
					26Y-1	KP310068			
					26Y-2	KP310069			
	-----	Lima, Peru	-----	-----	26YP-2	KP310070	Nie WZ	20140307	
					28Ae-1	KP310055			
					28Ae-2	KP310056			
					28Ae-3	KP310057			
					26D-1	KP310066			
	<i>Chrysomya pinguis</i>	Zhongshan	Guangdong	113.423	22.517	199C-1	KJ129510	Wei XY	20130304
						199C-2	KJ129511		
199C-3						KJ129512			
Fangchenggang		Guangxi	108.055	21.892	199D-1	KJ129513	Wang XD	20130407	
					199D-2	KJ129514			
					199D-3	KJ129515			
Zunyi		Guizhou	107.191	27.937	199RS-1	KJ129525	Liu DX	20130801	
					199RS-2	KJ129526			
					199RS-3	KJ129527			
Shijiazhuang		Hebei	114.353	37.909	199K-1	KJ129507	Liu DX	20120822	
					199K-2	KJ129508			
					199K-3	KJ129509			
Zhengzhou		Henan	113.673	34.900	199I-1	KJ129528	Chen J	20130812	
					199I-2	KJ129529			
					199I-3	KJ129530			
Jiaozuo			113.386	35.421	199J-1	KJ129531	Hu J	20130813	
					199J-2	KJ129532			
					199J-3	KJ129533			
Datong	Shanxi	113.142	40.114	199Aa-1	KJ129540	Qiu XY	20130809		
				199Aa-2	KJ129541				
				199Aa-3	KJ129542				
Panzhihua	Sichuan	101.636	26.711	199O-1	KJ129522	Liu DX	20130816		
				199O-2	KJ129523				
				199O-3	KJ129524				
Bayi, Nyingchi	Tibet	94.343	29.664	199E-1	KJ129534	Wu KL	20130702		
				199E-2	KJ129535				
				199E-3	KJ129536				
Paizhen, Nyingchi		94.389	29.623	199Ee-1	KJ129537	Liu DX	20130705		
				199Ee-2	KJ129538				
				199Ee-3	KJ129539				
				199Ee-4	KJ129516	Wu KL	20130705		
				199Ee-5	KJ129517				
				199Ee-6	KJ129518				
Motuo, Nyingchi		95.333	29.325	199F-1	KJ129519	Liu DX	20130709		
				199F-2	KJ129520				
				199F-3	KJ129521				
<i>Achoetandrus rufifacies</i>	Luan	Anhui	116.255	31.360	76C-1	KJ129261	Wang XD	20120903	
	Fuzhou	Fujian	119.290	26.093	76G-1	KJ129271	Wang XD	20120926	
					76G-2	KJ129272			
					76G-3	KJ129273			
	Xiamen		118.176	24.518	76E-1	KJ129265	Wang XD	20120918	
					76E-2	KJ129266			
					76E-3	KJ129267			
	Wuyishan		117.986	27.625	76F-1	KJ129268	Wang XD	20120925	
76F-2					KJ129269				

					76F-3	KJ129270			
	Shantou	Guangdong	116.712	23.403	76D-1	KJ129262	Yue QY	20120518	
					76D-2	KJ129263			
					76D-3	KJ129264			
	Yunfu		112.058	22.941	76H-1	KJ129274	Wang XD	20121021	
					76H-2	KJ129275			
					76H-3	KJ129276			
	Zhongshan		113.423	22.517	76A-1	KJ129257	Yue QY	20121023	
					76A-2	KJ129283			
					76A-3	KJ129284			
					76A-4	KJ129285			
	Haikou	Hainan	110.316	20.034	233C-1	KJ129289	Wang XD	20121126	
					233C-2	KJ129290			
					233C-3	KJ129291			
	Wuzhishan		109.523	18.789	233B-1	KJ129286	Wang XD	20121122	
					233B-2	KJ129287			
	Xiangyang	Hubei	112.109	32.035	76J-1	KJ129280	Hu J	20130819	
					76J-2	KJ129281			
					76J-3	KJ129282			
	Nanjing	Jiangsu	118.746	32.087	76B-1	KJ129258	Liu DX	20120829	
					76B-2	KJ129259			
					76B-3	KJ129260			
	Emeishan	Sichuan	103.493	29.591	76I-1	KJ129277	Liu DX	20130722	
					76I-2	KJ129278			
					76I-3	KJ129279			
<i>Protophormia terraenovae</i>	Zhongshan	Guangdong	113.423	22.517	97A-1	KJ129244	Guan W	20111201	
					97A-2	KJ129245			
					97A-3	KJ129246			
					97A-4	KJ129247			
	Hohhot	Inner Mongolia	111.229	41.323	97C-1	KJ129251	Qiu XY	20130805	
					97C-2	KJ129252			
					97C-3	KJ129253			
	Erlianhot		111.962	43.657	97D-1	KJ129254	Hu J	20130807	
					97D-2	KJ129255			
					97D-3	KJ129256			
	Paizhen, Nyingchi	Tibet	94.213	29.216	97B-1	KJ129248	Wu KL	20130703	
					97B-2	KJ129249			
					97B-3	KJ129250			
<i>Lucilia cuprina</i>	Chaohu	Anhui	117.872	31.642	174L-1	KJ129408	Wang XD	20121002	
					174L-2	KJ129409			
					174L-3	KJ129410			
	Luan		116.255	31.360	174F-1	KJ129392	Wang XD	20120903	
					174F-2	KJ129393			
					174F-3	KJ129394			
	Fuzhou	Fujian	119.315	26.053	174J-1	KJ129402	Wei XY	20120927	
					174J-2	KJ129403			
					174J-3	KJ129404			
					174H-2	KJ129398			
	Wuyishan		118.023	27.734	174I-1	KJ129399	Wei XY	20120922	
					174I-2	KJ129400			
					174I-3	KJ129401			
	Shantou	Guangdong	116.712	23.403	174B-1	KJ129386	Yue QY	20120518	
					174B-2	KJ129387			
					174B-3	KJ129388			
	Wuzhishan	Hainan	109.671	18.880	174M-1	KJ129411	Wang XD	20121123	
					174M-2	KJ129412			
					174M-3	KJ129413			
	Sanya		109.508	18.256	174N-1	KJ129414	Wang XD	20130507	
					174N-2	KJ129415			
					174N-3	KJ129416			
	Zhengzhou	Henan	113.685	34.762	174P-1	KJ129418	Qiu XY	20130812	
					174P-2	KJ129419			
	Xiaogan	Hubei	114.120	31.556	174K-1	KJ129405	Wei XY	20121001	
					174K-2	KJ129406			
					174K-3	KJ129407			
	Nanjing	Jiangsu	118.746	32.087	174E-1	KJ129389	Liu DX	20120829	

	Jinan	Shandong	117.025	36.675	174E-2	KJ129390	Wang XD	20120823
					174E-3	KJ129391		
					174G-1	KJ129395		
					174G-2	KJ129396		
					174O-1	KJ129417		
					174A-1	KJ129383		
<i>Lucilia illustris</i>	Jinan	Shandong	117.025	36.675	174A-2	KJ129384	Wang XD	20120816
					174A-3	KJ129385		
					236F-1	KJ129548		
	Jinan	Shandong	117.025	36.675	236F-2	KJ129549	Hu J	20130804
					236F-3	KJ129550		
					236D-1	KJ129545		
	Datong	Shanxi	113.294	39.581	236D-2	KJ129546	Wang XD	20120823
					236D-3	KJ129547		
					236G-1	KJ129551		
	Jixian	Tianjin	117.488	40.022	236G-2	KJ129552	Yue QY	20130810
					236A-1	KJ129543		
					236A-2	KJ129544		
<i>Lucilia sericata</i>	Chaohu	Anhui	117.673	31.431	56N-1	KJ129320	Wang XD	20121002
	Luan		116.255	31.360	56G-1	KJ129302	Wang XD	20120903
	Chongqing	Chongqing	106.429	29.821	56G-2	KJ129303	Liu DX	20130727
					56S-1	KJ129333		
					56S-2	KJ129334		
	Fuzhou	Fujian	119.315	26.053	56S-3	KJ129335	Wei XY	20120927
					56L-1	KJ129314		
					56L-2	KJ129315		
	Wuyishan		118.023	27.734	56L-3	KJ129316	Wang XD	20120924
					56H-1	KJ129305		
					56H-2	KJ129306		
	Shantou	Guangdong	116.712	23.403	56H-3	KJ129307	Yue QY	20120518
					56D-1	KJ129293		
					56D-2	KJ129294		
	Zhongshan		113.423	22.517	56D-3	KJ129295	Feng XM	20110318
					56A-1	KJ129292		
					56O-1	KJ129321	Wang XD	20130426
	Sanya	Hainan	109.508	18.256	56O-2	KJ129322		
					56O-3	KJ129323		
	Shijiazhuang	Hebei	114.411	38.070	56E-1	KJ129296	Wang XD	20120822
					56E-2	KJ129298		
					56J-1	KJ129308	Wang XD	20120918
	Qinhuangdao		119.485	39.835	56J-2	KJ129309		
					56J-3	KJ129310		
	Xiangyang	Hubei	112.178	32.045	56Y-1	KJ129339	Chen J	20130819
					56Y-2	KJ129340		
					56Y-3	KJ129341		
	Xiaogan		114.117	31.558	56M-1	KJ129317	Wei XY	20120930
					56M-2	KJ129318		
					56M-3	KJ129319		
	Jiaozuo	Henan	113.386	35.421	56W-1	KJ129336	Hu J	20130813
					56W-2	KJ129337		
					56W-3	KJ129338		
	Nanjing	Jiangsu	118.880	31.322	56F-1	KJ129299	Liu DX	20120829
					56F-2	KJ129300		
					56F-3	KJ129301		
	Jinan	Shandong	117.025	36.675	56Aa-1	KJ129311	Wang XD	20120823
					56Aa-2	KJ129312		
					56R-1	KJ129330	Wu KL	20130716
	Panzhihua	Sichuan	101.636	26.711	56R-2	KJ129331		
					56R-3	KJ129332		
	Lhasa	Tibet	91.087	29.656	56P-1	KJ129324	Liu DX	20130628
					56P-2	KJ129325		
					56P-3	KJ129326		
	Paizhen, Nyingchi		93.075	29.046	56Q-1	KJ129327	Liu DX	20130704
					56Q-2	KJ129328		

<i>Hemipysylla ligurriensis</i>	Luan	Anhui	116.333	31.393	56Q-3	KJ129329		
					144G-1	KJ129356	Wang XD	20120903
					144G-2	KJ129357		
					144G-3	KJ129358		
	Wuyishan	Fujian	118.023	27.734	83C-1	KJ129359	Wang XD	20120924
					83C-2	KJ129360		
					83C-3	KJ129361		
	Chongqing	Chongqing	106.429	29.821	144R-1	KJ129377	Liu DX	20130725
					144R-2	KJ129378		
					144R-3	KJ129379		
	Shantou	Guangdong	116.730	23.366	144C-1	KJ129344	Yue QY	20120517
					144C-2	KJ129345		
					144C-3	KJ129346		
	Shaoguan		113.587	24.864	144S-1	KJ129380	J. L. Liao	20130928
					144S-2	KJ129381		
					144S-3	KJ129382		
	Yunfu		112.059	22.912	144D-1	KJ129347	Hu J	20120907
					144D-2	KJ129348		
					144D-3	KJ129349		
	Zhanjiang		109.847	20.555	144A-1	KJ129342	Yue QY	20120509
					144A-2	KJ129343		
	Wuzhishan	Hainan	109.671	18.880	144O-1	KJ129362	Wang XD	20121123
					144O-2	KJ129363		
					144O-3	KJ129364		
					144O-4	KJ129368		
					144O-5	KJ129369		
					144O-6	KJ129370		
Sanya		109.508	18.256	144N-1	KJ129365	Wang XD	20121120	
				144N-2	KJ129366			
				144N-3	KJ129367			
Nanjing	Jiangsu	118.880	31.322	144F-1	KJ129353	Liu DX	20120829	
				144F-2	KJ129354			
				144F-3	KJ129355			
Taian	Shandong	117.093	36.311	144E-1	KJ129350	Wang XD	20120826	
				144E-2	KJ129351			
				144E-3	KJ129352			
Emeishan	Sichuan	103.493	29.591	144Q-1	KJ129374	Wu KL	20130722	
				144Q-2	KJ129375			
				144Q-3	KJ129376			
Nyingchi	Tibet	95.333	29.325	144P-1	KJ129371	Wu KL	20130709	
				144P-2	KJ129372			
				144P-3	KJ129373			
<i>Musca domestica</i>	Chaochu	Anhui	117.872	31.642	150M-1	KJ129454	Wang XD	20121002
					150M-2	KJ129455		
	Luan		116.255	31.360	150H-1	KJ129439	Wang XD	20120903
					150H-2	KJ129440		
					150H-3	KJ129441		
	Fuzhou	Fujian	119.315	26.053	150J-1	KJ129445	Wei XY	20120927
					150J-2	KJ129446		
					150J-3	KJ129447		
	Xiamen		118.110	24.465	150I-1	KJ129442	Wei XY	20120917
					150I-2	KJ129443		
					150I-3	KJ129444		
	Meizhou	Guangdong	116.182	23.740	150L-1	KJ129451	Qiu DY	20121002
					150L-2	KJ129452		
					150L-3	KJ129453		
	Shantou		116.712	23.403	150B-1	KJ129423	Yue QY	20120518
					150B-2	KJ129424		
					150B-3	KJ129425		
	Yunfu		112.059	22.912	150N-1	KJ129456	Yue QY	20121020
	Zhongshan		113.486	22.568	150A-1	KJ129420	Yang MF	20120809
				150A-2	KJ129421			
				150A-3	KJ129422			
Fangchenggang	Guangxi	108.055	21.892	150Q-1	KJ129462	Wang XD	20130407	
				150Q-2	KJ129463			
				150Q-3	KJ129464			

	Ledong	Hainan	108.863	18.740	150R-1	KJ129465	Wang	20130428
					150R-2	KJ129466	XD	
					150R-3	KJ129467		
	Haikou		110.316	20.034	150P-1	KJ129459	Wang	20121126
					150P-2	KJ129460	XD	
					150P-3	KJ129461		
	Wuzhishan		109.523	18.789	150O-1	KJ129457	Wang	20121122
					150O-2	KJ129458	XD	
	Shijiazhuang	Hebei	114.411	38.070	150E-1	KJ129432	Wang	20120823
					150E-2	KJ129433	XD	
	Qinhuangdao		119.485	39.835	150D-1	KJ129429	Wang	20120818
					150D-2	KJ129431	XD	
	Xiaogan	Hubei	114.120	31.556	150K-1	KJ129448	Wei XY	20120930
					150K-2	KJ129449		
					150K-3	KJ129450		
	Nanjing	Jiangsu	118.746	32.087	150G-1	KJ129436	Wang	20120831
					150G-2	KJ129437	XD	
					150G-3	KJ129438		
	Taian	Shandong	117.093	36.311	150F-1	KJ129434	Wang	20120826
					150F-2	KJ129435	XD	
	Jixian	Tianjin	117.274	40.106	150C-1	KJ129426	Liu DX	20120815
					150C-2	KJ129427		
					150C-3	KJ129428		
	-----	California, USA	-----	-----	150S-1	KJ129468	Yue QY	20120926
					150S-2	KJ129469		
					150S-3	KJ129470		
<i>Musca sorbens</i>	Luan	Anhui	116.333	31.393	98F-1	KJ129485	Wang	20120903
					98F-2	KJ129486	XD	
					98F-3	KJ129487		
	Fuzhou	Fujian	119.315	26.053	98I-1	KJ129494	Wei XY	20120927
					98I-2	KJ129495		
	Xiamen		118.197	24.441	98G-1	KJ129488	Wei XY	20120919
					98G-2	KJ129489		
					98G-3	KJ129490		
	Wuyishan		118.023	27.734	98H-1	KJ129491	Wei XY	20120925
					98H-2	KJ129492		
					98H-3	KJ129493		
	Shantou	Guangdong	116.717	23.372	98B-1	KJ129474	Yue QY	20120517
					98B-2	KJ129475		
	Yunfu		112.059	22.912	98D-1	KJ129479	Yue QY	20121020
					98D-2	KJ129480		
					98D-3	KJ129481		
	Zhongshan		113.333	22.303	98A-1	KJ129471	Huang YW	20111202
					98A-2	KJ129472		
					98A-3	KJ129473		
	Haikou	Hainan	110.316	20.034	98K-1	KJ129496	Wang	20121126
					98K-2	KJ129497	XD	
	Shijiazhuang	Hebei	114.411	38.070	98C-1	KJ129476	Wang	20120823
					98C-2	KJ129477	XD	
					98C-3	KJ129478		
	Xiangyang	Hubei	112.159	32.076	98M-1	KJ129501	Hu J	20130820
					98M-2	KJ129502		
					98M-3	KJ129503		
	Yueyang	Hunan	113.094	29.381	98N-1	KJ129504	Chen J	20130821
					98N-2	KJ129505		
					98N-3	KJ129506		
	Nanjing	Jiangsu	118.746	32.087	98Q-1	KJ129482	Wang	20120831
					98Q-2	KJ129483	XD	
					98Q-3	KJ129484		
<i>Graphomya rufitibia</i>	Chaohe	Anhui	117.673	31.431	163G-1	KJ129570	Wang	20121002
					163G-2	KJ129571	XD	
					163G-3	KJ129572		
	Huoshan		116.333	31.393	163F-1	KJ129567	Liu DX	20120903
					163F-2	KJ129568		
					163F-3	KJ129569		
	Fuzhou	Fujian	119.290	26.093	163L-1	KJ129585	Wei XY	20120926

					163L-2	KJ129586		
					163L-3	KJ129587		
	Wuyishan		117.986	27.625	163M-1	KJ129588	Wei XY	20120925
					163M-2	KJ129589		
					163M-3	KJ129590		
	Meizhou	Guangdong	116.182	23.740	163K-1	KJ129583	Qiu DY	20121002
					163K-2	KJ129584		
	Shantou		116.717	23.372	163Aa-1	KJ129553	Yue QY	20120517
					163Aa -2	KJ129554		
					163Aa -3	KJ129555		
	Yunfu		112.059	22.912	163E-1	KJ129564	Yue QY	20121020
					163E -2	KJ129565		
					163E -3	KJ129566		
	Fangchenggang	Guangxi	108.055	21.892	163I-1	KJ129576	Wang XD	20130407
					163I -2	KJ129577		
					163I-3	KJ129578		
	Guilin		110.253	25.915	163J-1	KJ129579	Wang XD	20130411
					163J -2	KJ129580		
					163J -3	KJ129581		
	Sanya	Hainan	109.508	18.256	163H-1	KJ129573	Wang XD	20121120
163H-2					KJ129574			
163H-3					KJ129575			
Yulongtan, Jinan	Shandong	117.025	36.675	163Ba-1	KJ129562	Wang XD	20120823	
				163Ba-2	KJ129563			
Daminghu, Jinan		117.015	36.666	163C-1	KJ129556	Wang XD	20120825	
				163C-2	KJ129557			
				163C-3	KJ129558			
				163C-4	KJ129559			
				163C-5	KJ129560			
Weinan	Shannxi	109.430	34.517	163C-6	KJ129561	Hu J	20130815	
				163O-1	KJ129591			
				163O-2	KJ129592			
<i>Sarcophaga albiceps</i>	Luan	Anhui	116.255	31.360	90B-1	KJ129151	Wang XD	20120903
					90B-2	KJ129152		
					90B-3	KJ129153		
	Fuzhou	Fujian	119.290	26.093	90K-1	KJ129167	Wei XY	20120926
					90K-2	KJ129168		
	Wuyishan		118.023	27.734	90L-1	KJ129169	Liu DX	20120924
	Meizhou	Guangdong	116.182	23.740	90Q-1	KJ129179	Yue QY	20121002
	Shantou		116.712	23.403	90H-1	KJ129164	Yue QY	20120518
	Shenzhen		114.550	22.533	90I-1	KJ129165	Wang XD	20120516
					90I-2	KJ129166		
	Yunfu		112.059	22.912	90G-1	KJ129161	Yue QY	20121020
					90G-2	KJ129162		
					90G-3	KJ129163		
	Zhanjiang		109.847	20.555	90F-1	KJ129159	Wang XD	20120508
					90F-2	KJ129160		
					90F-3	KJ129147		
	Wuzhishan	Hainan	109.671	18.880	90N-1	KJ129170	Wang XD	20121123
					90N-2	KJ129171		
					90N-3	KJ129172		
					90N-4	KJ129173		
	Qinhuangdao	Hebei	119.485	39.835	90A-1	KJ129148	Wang XD	20120918
					90A-2	KJ129149		
					90A-3	KJ129150		
	Nanjing	Jiangsu	118.880	31.322	90E-1	KJ129157	Liu DX	20120829
					90E-2	KJ129158		
	Taian	Shandong	117.093	36.311	90O-1	KJ129174	Wang XD	20120826
						90O-2		
	Jinan		117.025	36.675	90P-1	KJ129176	Wang XD	20120823
					90P-2	KJ129177		
		90P-3			KJ129178			
	Jixian	Tianjin	117.488	40.022	90D-1	KJ129154	Wang XD	20120816
					90D-2	KJ129155		

<i>Sarcophaga brevicornis</i>					90D-3	KJ129156		
	Luan	Anhui	116.333	31.393	208K-1	KJ129195	Wang	20120903
					208K-2	KJ129196	XD	
					208K-3	KJ129197		
	Fuzhou	Fujian	119.290	26.093	208N-1	KJ129200	Wei XY	20120926
					208N-2	KJ129201		
					208N-3	KJ129202		
	Wuyishan		118.023	27.734	208M-1	KJ129198	Wang	20120924
					208M-2	KJ129199	XD	
	Meizhou	Guangdong	116.141	24.316	208E-1	KJ129184	Yue QY	20121004
					208E-2	KJ129185		
					208E-3	KJ129186		
	Shenzhen		114.213	22.585	208C-1	KJ129182	Wang	20120514
					208C-2	KJ129183	XD	
	Zhongshan		113.423	22.517	208A-1	KJ129180	Wei XY	20130222
					208A-2	KJ129181		
	Haikou	Hainan	110.317	20.015	208Q-1	KJ129209	Wang	20121126
					208Q-2	KJ129210	XD	
					208Q-3	KJ129211		
	Sanya		109.508	18.256	208O-1	KJ129203	Wang	20121120
					208O-2	KJ129204	XD	
					208O-3	KJ129205		
	Wuzhishan		109.523	18.789	208P-1	KJ129206	Wang	20121122
					208P-2	KJ129207	XD	
					208P-3	KJ129208		
	Shijiazhuang	Hebei	114.353	37.909	208G-1	KJ129187	Wang	20120822
							XD	
	Nanjing	Jiangsu	118.880	31.322	208J-1	KJ129192	Liu DX	20120829
					208J-2	KJ129193		
					208J-3	KJ129194		
	Jinan	Shandong	117.025	36.675	208I-1	KJ129190	Wang	20120823
					208I-2	KJ129191	XD	
	Taian		117.093	36.311	208H-1	KJ129188	Wang	20120826
					208H-2	KJ129189	XD	
<i>Sarcophaga peregrina</i>	Fuzhou	Fujian	119.290	26.093	69M-1	KJ129239	Wei XY	20120926
					69M-2	KJ129240		
					69M-3	KJ129241		
	Xiamen		118.090	24.458	69K-1	KJ129233	Wang	20120903
					69K-2	KJ129234	XD	
					69K-3	KJ129235		
	Wuyishan		118.023	27.734	69L-1	KJ129236	Wang	20120924
					69L-2	KJ129237	XD	
					69L-3	KJ129238		
	Yunfu	Guangdong	112.059	22.912	69C-1	KJ129216	Yue QY	20120907
					69C-2	KJ129217		
					69C-3	KJ129218		
	Zhanjiang		109.847	20.555	69B-1	KJ129213	Yue QY	20120509
					69B-2	KJ129214		
					69B-3	KJ129215		
	Zhongshan		113.423	22.517	69A-1	KJ129212	Wei XY	20130222
	Shijiazhuang	Hebei	114.353	37.909	69E-1	KJ129222	Wang	20120822
					69E-2	KJ129223	XD	
					69E-3	KJ129224		
	Xiaogan	Hubei	114.120	31.556	69N-1	KJ129242	Wei XY	20120930
					69N-2	KJ129243		
	Nanjing	Jiangsu	118.880	31.322	69H-1	KJ129230	Liu DX	20120829
					69H-2	KJ129231		
					69H-3	KJ129232		
	Jinan	Shandong	117.025	36.675	69F-1	KJ129225	Wang	20120823
					69F-2	KJ129226	XD	
					69F-3	KJ129227		
	Taian		117.093	36.311	69G-1	KJ129228	Wang	20120826
					69G-2	KJ129229	XD	
	Jixian	Tianjin	117.488	40.022	69D-1	KJ129219	Wang	20120816
					69D-2	KJ129220	XD	
					69D-3	KJ129221		

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Figure S1 Maximum likelihood tree showing relationships between three calliphorid flies: *Chrysomya megacephala* (blue), *Chrysomya pinguis* (green), and *Protophormia terranova* (red), with *Achoetandrus rufifaces* assigned as the outgroup. The dataset included 53 unique *C. megacephala*, 13 *C. pinguis*, and 5 *P. terranova* COI barcode region sequences. Numbers above branches are bootstrap support (1000 replicates) for neighbor-joining distance analysis using a maximum likelihood distance model. See methods for more details. Both the maximum likelihood and neighbor-joining bootstrap analysis strongly support three distinct clades, one for each species.