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Cytochrome oxidase II gene "short fragment" applicability in identification of forensically important insects

Sanaa Mohamed Aly[1, 2], Jifang Wen[1], Xiang Wang[1], Jifeng Cai[1]*

Abstract: Correct insect identification is an important issue in the forensic entomological analysis. Short DNA segments can be used to uniquely identify species. In order to be useful as a forensic tool, a short DNA sequence must be not only easily sequenced, but also contains enough variation to generate unique identifiers at either the species or population levels. In the present study, we assessed the applicability of a 189-bp mitochondrial cytochrome oxidase subunit II (COII) gene fragment in identification. We analyzed 53 specimens representing 14 species belong to Sarcophagidae [Sarcophaga carnaria, Sarcophaga dux, Sarcophaga albiceps, Helicophagella melanura, Phallosphaera gravelyi, Boettcherisca peregrina and Wohlfahrtia nuba], Calliphoridae [Lucilia cuprina, Lucilia sericate, Chrysomya megacephala, Chrysomya rufifacie and Chrysomya albiceps] and Muscidae [Musca domestica and Musca autumnalis]. This region was amplified using polymerase chain reaction (PCR) followed by direct sequencing of the amplification products. Nucleotide sequence divergences were calculated using the Kimura two-parameter (K2P) distance model and a neighbour-joining (NJ) phylogenetic tree generated. No intraspecific variation within same species (0%), while interspecific variation ranged from 2% to 25%. All specimens were properly assigned into correct species apart from H. melanura and M. autumalis. Although molecular methods are very useful to identify forensically important insect, we propose to use it only in addition to the conventional methods.

Key Words: Forensic entomology; Cytochrome oxidase II; Diptera; Identification.

The use of insects can specify the minimum postmortem interval (PMImin) in forensic investigations [1,2]. Species identification of forensically important insects is a very complicated and difficult process, in which the small anatomic identification keys are observed with a stereomicroscope [3]. Moreover, most insect specimens recovered from the scene are immature stages, such as eggs, larvae, or pupae, which are in many instances indistinguishable among different species [4]. Consequently, proper species identification and PMImin estimation is almost too difficult.

Deoxyribonucleic acid (DNA)-based methods for species identification may solve these problems especially for scientists not formally trained in taxonomy and can be applied on all life stages including ancient or damaged samples when anatomical characteristics were destroyed [5,6]. Mitochondrial DNA (mtDNA) is preferably applied for forensic investigations because greater abundance in tissues, when compared with nuclear DNA (nuDNA), makes it easier for extraction even from small amount of sample. In addition, because of its strictly maternal inheritance and no genetic recombination, mtDNA haplotype is a good candidate for evolutionary and population genetics study [5,7,8].

Mitochondrial cytochrome oxidase I, II (COI, COII) genes are suitable as molecular markers because relatively a high degree of genetic variation in this region has been reported [5,7]. Currently, COI has been selected as a standard barcode gene for animal groups.

However, the rationale of selection of COI as

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1) PhD, Department of Pathology and Forensic Science , School of Basic Medical Sciences, Central South University, Changsha 410013, Hunan, China
**Corresponding author: Department of Pathology and Forensic Science, School of Basic Medical Sciences, Central South University, Changsha 410013, Hunan, China, E-mail: sasydayem@hotmail.com, Tel.: +86 731 82355414; Fax: +86 731 82355414*
2) PhD, Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Suez Canal University, Ismailia 41522, Egypt;

standard barcode is subject to debate, and scientists have found its less effective in some taxon groups [9-12]. The search for the most suitable gene for species identification is not over, with several recent studies testing the efficiencies of different genes, using part of, or the whole of mtDNA genome to look for the optimal DNA barcode gene [13,14].

Many studies evaluate COI and COII together as identification marker [4,5,15-19]. Although COII gene is useful in evolution studies, population genetics and systematics due to the relatively high degree of variation in the region [20], but there are few publications on the molecular identification of forensically relevant flies based on COII alone [21,22]. Whereas longer DNA fragments may minimize stochastic variation across taxa and be more reliable, shorter ones have many advantages, such as quick, easy and economical. This rational put short fragment as high candidate for using as forensic identification marker.

Therefore, the aim of our study was to investigate the applicability of 189-bp COII fragment in identification of common forensically important dipteran flies originating from Egypt and China as a step toward finding optimal DNA fragment for species identification.

Materials and methods

1. Specimens

A total of 53 specimens were analyzed, representing 14 species which included 5 species of Calliphoridae, 7 species of Sarcophagidae and 2 species of Muscidae. All samples were collected using traps baited with animal remains (rabbit, dog, or pig) from different locations. Samples were subsequently air-dried at room temperature or stored in 70% ethanol at −20oC. Egyptian flies were trapped in Suez Canal region (northeastern Egypt). Chinese specimens were captured from Changsha (central southern China).

Third instar larva, pupae and adults were used to simulate entomological evidence that could be collected from scene. Laboratory rearing of immature samples were conducted to either to diagnose or confirm correct species diagnosis. Origins and details of specimens are shown in Table 1. All samples were identified using morphological keys with the assistance of entomologists from faculty of agriculture- Suez Canal University- Egypt and Hunan Agricultural University- China [23-27].

2. DNA extraction

MtDNA from all samples was extracted using QIAamp DNA Mini Tissue Kit (Qiagen, Germany). To avoid possible contamination of fly DNA with DNA from ingested proteins and eggs of gut parasites, the thoracic muscle of each insect was used as the source of DNA, whereas the head and abdomen were retained for further morphological and molecular analysis.

3. PCR amplification

A portion of 189-bp fragment of the mitochondrial COII gene was amplified and sequenced by using forward primer (5'-ATTAGATGTTGATAATCG-3') and reverse primers (5'-ACAAATTTCTGAACATTG-3').

The PCR reaction volume was 25µl, containing 1-5µl (20-40ng) of template DNA, 12.5µl 2xGoTaq® Green Master Mix (4µl dNTP (1mmol/ml), 1.0u Taq polymerase, 2.5µl 10xbuffer ($Mg2+$ 1.5mmol/l)), 0.25-2.5µl each primer (10µM), Nuclease-Free Water added to a total volume of 25 µl. PCR amplifications were performed in a Thermo Cycler (Perkin-Elmer 9600) and programmed with the following parameters: initial step at 94°C (3 min), continued for 30 cycles of 94°C (30s) and 50°C (30s for mtDNA annealing) and 72°C (30s). An elongation of PCR products by 72°C for 5min completed the reaction.

4. Sequencing

1% agarose gel electrophoresis was used to isolate PCR products, which were then purified using a Qiaquick PCR Purification kit (Qiagen, Germany). Columns cycle sequencing was performed on both forward and reverse strands using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit by ABI PRISM 3730 (Applied Biosystems, Foster City, USA) with BigDye terminator v3.1 as the sequencing agent. Sequence chromatograms were edited, and discrepancies between forward and reverse sequences were resolved using Sequence Navigator (v1.01, Applied Biosystems, Foster City, USA).

5. COII sequencing analysis and phylogenetic tree construction

Since the sequences were protein coding and did not contain any insertions or deletions, all resultant sequences in this study were aligned using ClustalW. To identify species, the COI sequences were compared with Dipteral sequences on the NCBI web site via the BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) function. The sequences were tested using MEGA version 5.03 [28]. Phylogenetic tree based on the 189-bp COII fragment was constructed by neighbour-joining (NJ) method using Kimura two-parameter (K2P) model implemented in the MEGA5 and the tree was tested by 1000 bootstrap replicates.

Results and Discussion

The focus of this study was to develop a forensic tool and evaluate the potential value of small COII fragment for basic biological studies of these insects from Egypt and China. To date, there is no publication on the molecular identification of forensically relevant flies from Egypt. The research by Guo et al. (2010) deals with the molecular analysis of 3 Sarcophaginae species (Boerttcherisca peregrina, Parasarcophaga albiceps and Parasarcophaga dux) frequently found on carrion. We extend this research by including additional common forensically important fly (Diptera) species from 2 different geographical areas [22].

Sequences from 53 flies were successfully sequenced and aligned. The sequences corresponded to positions 108–295 of Drosophila yakuba (GenBank accession number DQ382818.1). No insertions or deletions were identified within the aligned sequences

Table 1. Reference data of specimens used in the present study.

Sample	Species	Type of specimen	Location	Code in phylogenetic tree
1	*Sarcophaga carnaria* (Linnaeus 1758)	Pupa	Ismailia, Egypt	S.carnaria1
2		Pupa	Ismailia, Egypt	S.carnaria2
3		Pupa	AlKantara Shark, Egypt	S.carnaria3
4		Adult/ ♀	Portsaid, Egypt	S.carnaria4
5		Adult/ ♀	Ismailia, Egypt	S.carnaria5
6		Adult/ ♂	Ismailia, Egypt	S.carnaria6
7		Adult/ ♂	AlKantara Shark, Egypt	S.carnaria7
8		Adult/ ♂	Portsaid, Egypt	S.carnaria8
9		Adult/ ♂	Suez, Egypt	S.carnaria9
10	*Sarcophaga dux* (Thompson, 1869)	Adult/ ♂	Changsha, China	S.dux1
11		Adult/ ♂	Changsha, China	S.dux2
12	*Sarcophaga albiceps* (Meigen, 1826)	Adult/ ♂	Changsha, China	*S.albiceps1*
13		Adult/ ♂	Changsha, China	*S.albiceps2* *S.albiceps3*
14		Adult/♀	Changsha, China	
15	*Wohlfahrtia nuba* (Wiedemann, 1830)	Adult/ ♂	Ismailia, Egypt	W.nuba1
16		Adult/ ♂	Ismailia, Egypt	W.nuba2
17		Adult/ ♂	Portsaid, Egypt	W.nuba3
18		Larva	Ismailia, Egypt	W.nuba4
19		Larva	Ismailia, Egypt	W.nuba5
20		Larva	Portsaid, Egypt	W.nuba6
21		Larva	Ismailia, Egypt	W.nuba7
22	*Helicophagella melanura (Meigen, 1826)*	Adult/♂	Changsha,China	*S.melanura1*
23		Adult/♂	Changsha,China	*S.melanura2*
24		Adult/♀	Changsha,China	*S.melanura3*
25	*Phallosphaera gravelyi (Senior-White, 1924)*	*Adult/♂*	China	*P. gravelyi 1*
26		Adult/♀	China	*P. gravelyi2*
27		Adult/♀	China	*P. gravelyi3*
28	*Boettcherisca peregrina* (R-D, 1830)	Adult/♂	Changsha, China	*B. peregrine1*
29		Adult/♀	Changsha, China	*B. peregrine2*
30		Adult/♀	Changsha, China	*B. peregrine3*
31	*Lucilia cuprina* (Wiedemann, 1830)	Adult/♂	Changsha, China	*L. cuprina1*
32		Adult/♀	Changsha, China	*L. cuprina2*
33		Adult/♀	Changsha, China	*L. cuprina3*
34		Adult/♀	Changsha, China	*L. cuprina4*
35	*Lucilia sericata* (Meigen, 1826)	Adult/♀	Portsaid, Egypt	*L. sericata1*
36		Adult/♀	Ismailia, Egypt	*L. sericata2*
37		Adult/♂	Suez, Egypt	*L. sericata3*
38		Adult/♂	Ismailia, Egypt	*L. sericata4*
39	*Chrysomya megacephala (Fabricius,1794)*	Adult/♂	Changsha, China	*C. megacephala1*
40		Adult/♂	Changsha, China	*C.megacephala2*
41		Adult/♂	Changsha, China	*C.megacephala3*
42	*Chrysomya rufifacies* (Macquart, 1842)	Adult/♀	Changsha, China	*C. rufifacies1*
43		Adult/♀	Changsha, China	*C. rufifacies2*
44		Adult/♀	Changsha, China	*C. rufifacies3*
45		Adult/♂	Changsha, China	*C. rufifacies4*
46	*Chrysomya albiceps* (Wiedemann, 1819)	Adult/♀	Changsha, China	*C. albiceps1*
47		Adult/♀	Changsha, China	*C. albiceps2*
48		Adult/♂	Changsha, China	*C. albiceps3*
49		Adult/♂	Changsha, China	*C. albiceps4*
50	*Musca domestica* (Linnaeus, 1758)	Adult/♀	Ismailia, Egypt	*M. domestica1*
51		Adult/♀	Suez, Egypt	*M. domestica2*
52	*Musca autumnalis* (De Geer, 1776)	Adult/♂	Changsha, China	*M. autumnalis1*
53		Adult/♂	Changsha, China	*M. autumnalis2*

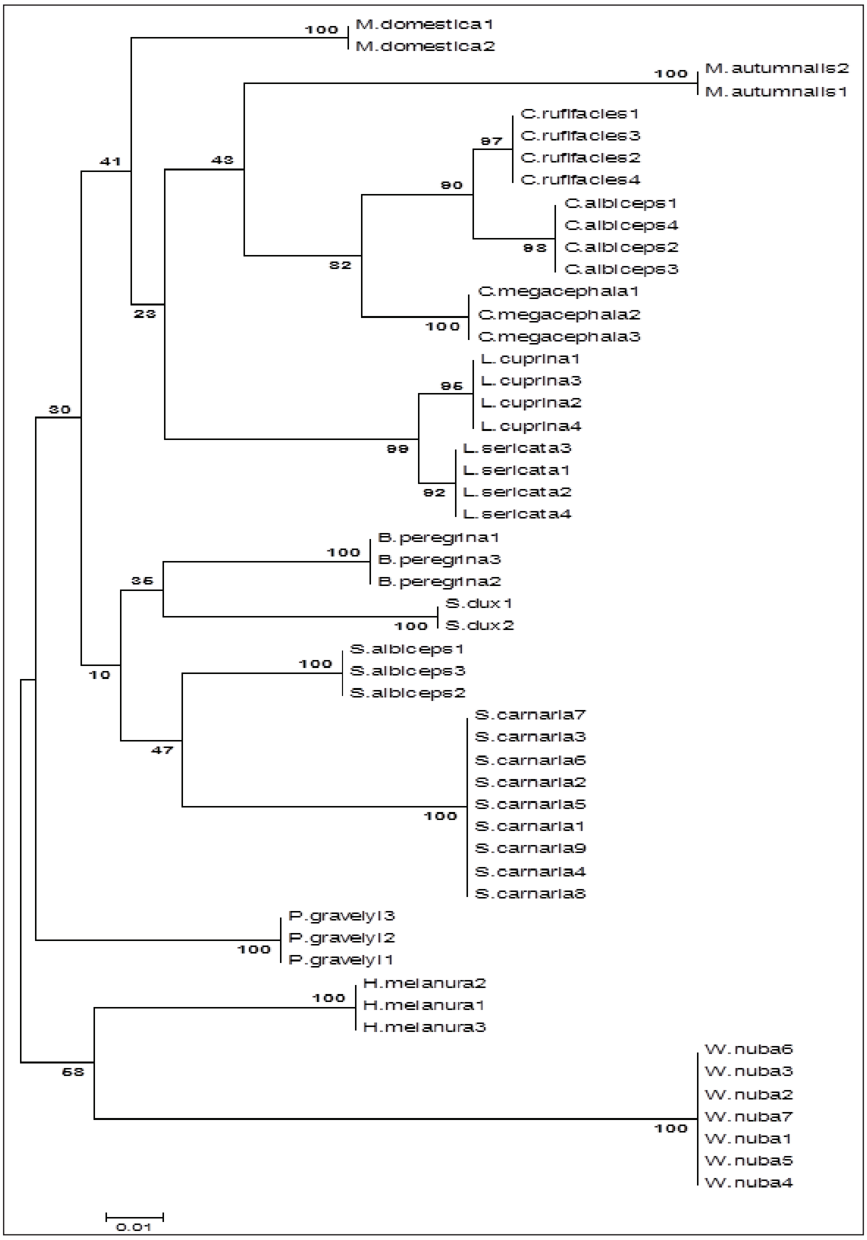

Figure 1: Neighbour-joining (NJ) tree of Kimura two- parameter (K2P) distances for 53 cytochrome oxidase subunit II (COII) gene sequences from 14 species of Egyptian and Chinese Diptera of forensic interest. Numbers on branches indicate the support value. Evolutionary distance divergence scale bar is 0.01.

as reported by other studies conducted on other mtDNA fragments [29-32]. Of 189-bp analyzed, 62 of these positions were variant and parsimony-informative characters. As expected, this region of mtDNA was observed to have a strong AT bias (65%), which is characteristic of insect mtDNA [33] where the nucleotide compositions were T (32.7%), C (17%), A (32.3%) and G (18%).

NJ analysis was conducted to display relationships between the analyzed species. The NJ tree and sequence divergences are shown in Figure 1 and Table 2, respectively. All flies were rightly assigned into 14 species with monophyletic separation in the NJ tree. At species level, the full bootstrap values (100%) provide percentage robust support for the monophyly of S.carnaria, W. nuba, S. dux, S. albiceps, S. peregrine, C. megacephala, M. domestica and M. autumnalis.

The remaining tested species have high bootstrap values ranging from 91 to 99%. At subfamily level, Chrysomyinae (C.albiceps, C.rufifacies and C.megacephala) clustered together in one clade with 82% bootstrap support. Luciliinae (L. sericata, L. cuprina) clustered together in one clade with 90% bootstrap support. Although S.carnaria, S. dux, S. albiceps and B. peregrina clustered together in one clade but with very low bootstrap support (10%). P. gravelyi did not cluster with other tested Sarcophaginae but were closer to this clade than other subfamilies clades. H. melanura (Subfamily: Sarcophaginae) clustered together with W. nuba (Subfamily: Paramacronychiinae).

At family level, M. autumnalis (Family: Muscidae) clustered together with Chrysomyinae (Family: Calliphoridae) clade. This may be due to insufficient sequence variation within the tested COII fragment. The 2 tested Muscidea did not cluster together indicating the ability of this short COII fragment to identify the species from the same subfamily was not as efficient as that of the long fragments. All species correctly assigned to their subfamily and family except H. melanura and M. autumnalis.

Interestingly, this study shows that both sister species, L. sericata and L. cuprina, can be separated without bias with 99% bootstrap support, although both species could not be unambiguously identified in the past based on longer (635-bp) COII fragment [21].

All tested species displayed no intraspecific variation as shown in Table 2. The reason might be that the amplicon size of the COII sequence is too small to supply a sufficient amount of information and/ or sampling from 2 limited geographical areas so that sampling from further areas are required..

The range of interspecific variation was between 2% (L. cuprina to L. sericata) and 25% (W. nuba to M. autumnalis). Within the Calliphoridae, the 3 species of the genus Calliphora differed in no more than 5%. All 7 species belong to Sarcophagidae revealed a maximum interspecific nucleotide divergence of 21%. The most closely related Sarcophagidae species were S.carnaria, S.dux and S. albiceps (divergence of 7-12%). Within the Muscidae, the tested 2 species differed in no more than 12%. There is no overlapping between maximum intraspecific and minimum interspecific nucleotide divergence which help in correct identification of species. Hebert et al. (2003) suggested that intraspecific variation should not exceed 3% while between-species separation needs to exceed 3% [33].

Table 2. Calculated interspecific divergences expressed as percentage of total 189 bp of COII using neighbor-joining (NJ) approach with Kimura's 2-parameter (K2P) model.

No	Species	N*	$V^{\dagger}$	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	***L. cuprina***	**4**	**0**	**-**													
2	***L.sericata***	**4**	**0**	**2**	**-**												
3	***C. megacephala***	**3**	**0**	**12**	**13**	**-**											
4	***C. rufifacies***	**4**	**0**	**10**	**11**	**5**	**-**										
5	***C. albiceps***	**4**	**0**	**13**	**11**	**5**	**2**	**-**									
6	***S.carnaria***	**9**	**0**	**15**	**15**	**13**	**14**	**15**	**-**								
7	***S. dux***	**2**	**0**	**13**	**13**	**13**	**14**	**16**	**12**	**-**							
8	***S. albiceps***	**3**	**0**	**11**	**11**	**11**	**13**	**14**	**8**	**8**	**-**						
9	***S. melanura***	**3**	**0**	**14**	**13**	**14**	**16**	**17**	**12**	**12**	**12**	**-**					
10	***S.gravelyi***	**3**	**0**	**12**	**11**	**13**	**15**	**14**	**11**	**10**	**8**	**10**	**-**				
11	***B. peregrine***	**3**	**0**	**11**	**12**	**10**	**13**	**15**	**11**	**8**	**7**	**10**	**11**	**-**			
12	***W.nuba***	**7**	**0**	**19**	**20**	**19**	**20**	**20**	**21**	**20**	**19**	**15**	**17**	**19**	**-**		
13	***M. autumnalis***	**2**	**0**	**14**	**15**	**15**	**12**	**12**	**16**	**17**	**17**	**21**	**17**	**15**	**25**	**-**	
14	***M. domestica***	**2**	**0**	**10**	**10**	**10**	**11**	**13**	**13**	**11**	**10**	**12**	**12**	**9**	**15**	**12**	**-**

N* Number of specimens / $V^{\dagger}$ Intraspecific variations.

The 3% threshold has also been used by Meiklejohn et al. (2011) on Sarcophagidae barcode research [32]. Hebert et al. (2003) suggested using a standard COI threshold with care and establish group-specific thresholds [34]. Boehme et al. (2011) supported the idea of establishing group-specific thresholds because 3% will not be sufficient to differentiate Lucilia sister species which is in consistence with the present study results [2].

Conclusion

Short mtDNA fragments are high candidates for using as forensic identification markers to uniquely identify an unknown specimen to correct species. In this study, 189-bp COII sequence was assessed as a potential marker for the identification of common forensically important flies from Egypt and China. Because analysis based on 189-bp COII is in agreement with those based on anatomy except in H. melanura and M. autumnalis, we suggest to be used only in combination with conventional method. Further studies for other mtDNA fragment, other species from other parts of Egypt and China should be studied and local databases are strongly recommended.

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