Tropical Biomedicine 26(2): 173–181 (2009)

Sequence variation in the *cytochrome oxidase subunit I* and *II* genes of two commonly found blow fly species, *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) in Malaysia

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Received 4 February 2009; received in revised from 9 May 2009; accepted 15 May 2009

Abstract. The mitochondiral DNA region encompassing the *cytochrome oxidase subunit I* (COI) and *cytochrome oxidase subunit II* (COII) genes of two Malaysian blow fly species, *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) were studied. This region, which spans 2303bp and includes the COI, tRNA leucine and partial COII was sequenced from adult fly and larval specimens, and compared. Intraspecific variations were observed at 0.26% for *Ch. megacephala* and 0.17% for *Ch. rufifacies*, while sequence divergence between the two species was recorded at a minimum of 141 out of 2303 sites (6.12%). Results obtained in this study are comparable to published data, and thus support the use of DNA sequence to facilitate and complement morphology-based species identification.

INTRODUCTION

Analysis of insect succession pattern on various stages of corpse decomposition may facilitate criminal investigation through the estimation of the post-mortem interval (PMI), and sometimes may even provide clues to the cause of death (Anderson, 2004). Accurate species identification is therefore crucial especially when legal matters are involved (Wells & LaMotte, 2001).

Conventionally, adult insect species are identified based on specific morphological features, such as presence and number of bristles, wing venation, and body colouration. (Smith, 1986; Wallman & Donnellan, 2001). The immature stages are, however, almost impossible to identify and require trained eyes as identification is based on specific characters such as the pattern differences of the spine, posterior spiracle and cephalopharyngeal skeleton (Wells & Sperling, 1999, 2001; Nelson *et al.*, 2008). For most cases, species identification for the larval stages only becomes feasible when they reach the third instar (Wells *et al.*, 1999; Turchetto *et al.*, 2001). Younger larva requires rearing to adulthood and often involved additional work and time (Wallman & Adams, 2001).

The use of comparative DNA sequence analysis to facilitate species identification has become increasingly popular in recent years due to their ease of use, rapidity and

reliability (Vincent *et al.*, 2000; Wallman & Donnellan, 2001; Harvey *et al.*, 2003a, 2003b; Ames *et al.*, 2006; Nelson *et al.*, 2007, 2008). It is an attractive alternative to conventional morphology-based identification methods as it can be applied to any life stage and any preservation method of an insect (Sperling *et al.*, 1994). In line with DNA barcoding efforts (Hebert *et al.*, 2003; Hebert & Gregory 2005), mitochondrial DNA has been one of the more common targets for analysis, and has shown promising results in several forensic identification studies (Wells *et al.*, 2001; Harvey *et al.*, 2003b). Due to its higher sequence variability as compared to nuclear DNA (Moriyama & Powell, 1997), mitochondrial DNA is also widely used for evolutionary studies, species differentiation (Malgorn & Coquoz, 1999; Wells & Sperling, 2001; Zehner *et al.*, 2004), as well as intra- and interspecific comparison.

In Malaysia, forensic entomology is gradually gaining importance. Calliphoridae is the most important family involved in forensic related cases in Malaysia (Hamid *et al.*, 2003; Lee *et al.*, 2004) and these include *Chrysomya megacephala* (Fabricius), *Chrysomya rufifacies* (Macquart), *Chrysomya villenuevi* Patton, *Chrysomya nigripes* Aubertin, *Chrysomya bezziana* Villenueve and *Chrysomya pinguis* (Walker). Lee *et al.* (2004) reported the identification of several forensically important specimens collected from cases involving humans, which included families of Calliphoridae, Sarcophagidae, Muscidae and Stratiomyidae, Pthiridae and order of Coleoptera. It is anticipated that DNA-based analysis will accelerate the pace of species identification and discovery, and contribute to the speedy development of forensic entomology in this country.

In this paper, we evaluate the use of DNA-based analysis for the identification of two commonly found blowfly species, *Ch. megacephala* and *Ch. rufifacies* (Diptera: Calliphoridae) in Malaysia. This study should provide the much needed groundwork towards establishing the use of molecular tools for forensic entomology in this country.

MATERIALS AND METHODS

Fly and larval specimens

Live blow flies and immature stages (larvae and egg) were collected from several locations (Table 1). Live flies were collected from the field using meat baits. The immature stages of identified flies (egg, larva, pupa, puparium) were obtained from existing *Ch. megacephala* laboratory colony. Identification of adult flies was carried out according to the identification keys from Kurahashi *et al.* (1997). Fly specimens from archived material were also included in this study to compare the efficiency of DNA extraction and amplification. For DNA extraction of adults, only legs from one side of the flies were used while the remaining

Table 1. *Chrysomya megacephala* and *Chrysomya rufifacies* specimens used in this study

Species	Collection locality	Voucher
Ch. megacephala	Mortuary, University Malaya Medical Center	CM3, CM4, CM5
	Rimba Ilmu, University of Malaya	UM1, UM3, UM5
	Petaling Jaya, Selangor	Egg, Chrys
	Taman Melawati, Selangor	Fly
	Gombak, Selangor	CM41, CM43
	Muar, Johore	CM-Muar
Ch. rufifacies	Mortuary, University Malaya Medical Center	CR3, CR5, CR6
	Rimba Ilmu, University of Malaya	LSP2
	Petaling Jaya, Selangor	U5, U7

parts of the flies were maintained as voucher specimens. For large larvae, the middle third of the body was used for DNA extraction and the posterior and anterior ends were used as vouchers.

DNA extraction

Two legs were used for each fly sample. Larval specimens were soaked in warm water (60ºC) for 10 minutes prior to DNA extraction. Fly or larva tissues were placed in 1.5ml microfuge tubes, immersed briefly in liquid nitrogen, and then ground into powder using sterile plastic pestles. Total genomic DNA was then extracted using QIAamp® DNA Mini Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions with some modifications. After overnight incubation in ATL buffer (Qiagen), the samples were treated with RNaseA. At the end of the extraction process, the DNA was eluted once with 200µL of elution buffer, and eluted again with another 200µL after leaving to stand in elution buffer for 5 minutes.

PCR amplification

PCR amplification mixtures were prepared to contain the following: 100ng template DNA, 1 unit of *Taq* DNA polymerase (Promega, USA), 1 x PCR reaction buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 200 µM of each dNTPs (Promega) and 0.4 µM of each forward and reverse primers (Research Biolabs Technology, Singapore). Amplification reactions were performed in MJ PTC-200 thermal cycler.

Three sets of primers were used in this study, and were designed based on the description of Sperling *et al.* (1994) (Table 2). Relative positions and orientation of primers are shown in Figure 1. PCR parameters included an initial denaturation step of 94ºC of 5 minutes, followed by 35 cycles of 94ºC for 1 minute, 46ºC (TY-J-1460 & C1-N-2800), 58ºC (C1-J-2495 & TK-N-3775) and 45ºC (C1-J-2495 & C1-N-2800) for 1 minute 30 seconds and 72ºC for 2 minutes, followed by a final elongation step of 72ºC for 5 minutes. The PCR products were separated electrophoretically on 1% agarose gel (Promega) and visualized after ethidium bromide staining.

Purification of PCR products

PCR products were purified prior to cloning or direct sequencing. PCR products were purified using either the QIAquick® PCR Purification Kit or QIAquick® Gel Extraction Kit (Qiagen), and was performed according to the manufacturer's protocols. The successes of PCR products purification were confirmed by agarose gel electrophoresis.

Table 2. Primer sequences used to amplify overlapping segments of the mitochondrial COI and COII genes (Sperling *et al.*, 1994)

Primer ID	Sequence (5' – 3')
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC
C1-N-2800	CATTTCAAGCTGTGTAAGCATC
C1-J-2495	CAGCTACTTTATGAGCTTTAGG
TK-N-3775	GAGACCATTACTTGCTTTCAGTCATCT

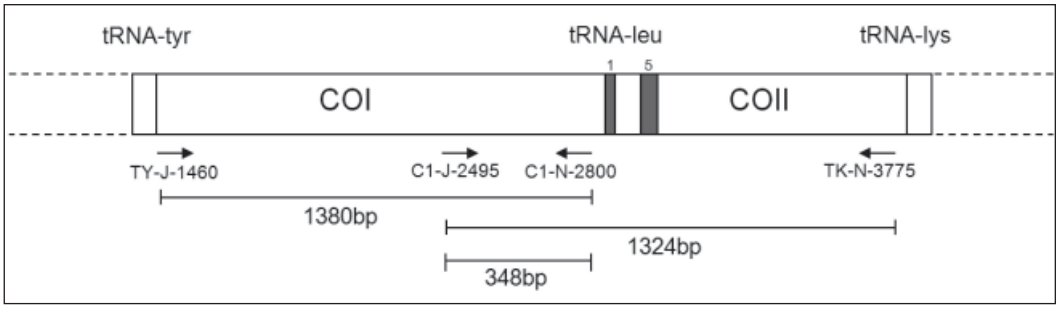

Figure 1. Schematic representation of the mitochondrial COI, COII, tRNA leucine genes and intergenic regions modified from Schroeder *et al.*, 2003. Shaded boxes (and corresponding numbers) represent non-coding nucleotides that are present between the genes. Locations of the primers and sizes of the amplification fragments using different primer combinations are shown.

Cloning and Sequencing

Purified PCR products were then cloned into the pGEM®-T Easy vector system (Promega) to facilitate DNA sequencing procedures. Sequencing was performed using ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. All samples were sequenced for both forward and reverse DNA strands using the universal M13 forward and reverse primers. Electrophoresis and detection of the sequencing reaction products was carried out in the capillary electrophoresis system ABI PRISM 3730xl capillary DNA Sequencer with a capillary length of 80 cm.

Data Analysis

DNA sequence chromatograms were read and discrepancies between forward and reverse sequences were resolved using the Chromas software version 2.33 (http://www.technelysium.com.au/chromas.html). Sequences from different specimens were aligned and phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 (Tamura *et al.*, 2007). Neighbour-joining, UPGMA and maximum parsimony analyses were used for phylogenetic analysis to compare the results of both distance and discrete methods. Both neighbour-joining and UPGMA analysis were constructed using the Kimura-2-Parameter model of nucleotide substitution and bootstrapping (n=1000). Whereas for the maximum parsimony tree search option, close-neighbour-interchange with search level 3 method was selected and bootstrapped for n=1000. A sequence from a species of the Sarcophagidae family was used as outgroup.

RESULTS AND DISCUSSION

As shown in Figure 2, DNA-based analyses can be applied to fresh as well as archived specimens, and also to various stages of the fly life cycle. As expected, analyses of the archived samples represented a greater challenge, as the bulk of the DNA from specimens of this age would have degraded through time. DNA analysis success rates will be improved however, if the PCR is limited to targeting smaller regions such as the 348bp COI fragment (amplified by primers C1-J-2495 & C1-N-2800), as the chances of obtaining intact mitochondrial fragment for amplification will increase (Sperling *et al.*, 1994). However, it is important to realize that while limiting the target region would facilitate rapid species identification in some cases, its use in phylogenetics and systematics studies would be compromised as the number of informative sites would be significantly reduced.

We had sequenced a total 2303bp of the complete coding sequence of *cytochrome oxidase subunit I* gene, complete coding sequence of tRNA-leucine and partial coding sequence of *cytochrome oxidase subunit II* for 12 specimens of *Ch. megacephala* and 6 specimens of *Ch. rufifacies*. These 2303bp sequences corresponded to positions 1461 to 3763 of *Drosophila yakuba* (Accession number NC_001322: Clary & Wolstenholme, 1985). Sequence analysis of the 18 specimens agreed with published data for insect mitochondrial DNA in that there was a strong AT bias at approximately 70.5% (Tamura, 1992; Crozier & Crozier, 1993; Simon *et al.*, 1994). Alignment and verification of the COI and COII sequences in both species showed no insertions or deletions but only substitutions. Throughout the 2303bp sequences, *Ch. rufifacies* displayed a transitions rate of 6.5 times higher than transversions (Table 3) while *Ch. megacephala* displayed a transitions rate of 2 times higher than transversion (Table 4). Verified sequences were submitted to the GenBank® (National Institutes of Health (NIH) Genetic Sequence database) with the accession numbers of AY909052-AY909055 (http://www.ncbi.nlm.nih.gov/).

Among the 12 sequences obtained for *Ch. megacephala*, 8 unique haplotypes were observed whilst all of the 6 sequences for *Ch. rufifacies* were unique haplotypes. Intraspecific variation levels for both *Ch. megacephala* and *Ch. rufifacies* were

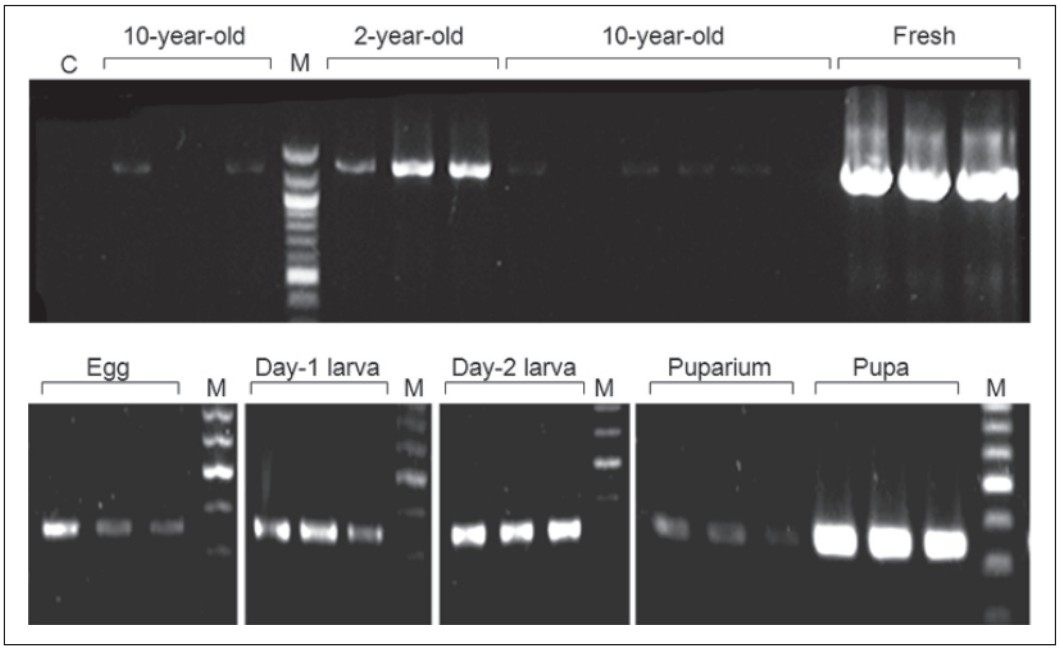

Figure 2. PCR results showing DNA of sufficient quantity and quality can be obtained from specimens of various sources and conditions.
(Top row) Results indicate that extraction and amplification were relatively more successful for fresh samples (bright bands) compared to older/archived specimens (faint bands). PCR amplification was carried out using TY-J-1460 and C1-N-2800 primers, with an expected product of 1380 bp. C, negative control; M, 100bp molecular weight marker (New England Biolabs, UK).
(Bottom row) Results indicate that the immature stages of flies are also amenable for DNA extraction and PCR. PCR amplification was carried out using C1-J-2495 and C1-N-2800 primers, with an expected product of 348 bp. M, 100bp molecular weight marker (New England Biolabs, UK).

Table 3. All DNA substitution sites that varied between local and foreign *Chrysomya rufifacies* [AY909055 and AF083658]. Nucleotide position numbers correspond to the investigated regions in this study

Species (Accession number)	Nucleotide Position																	
										1	1	1	1	1	1	2	2
	1	2	3	3	4	6	7	7	8	0	2	3	3	8	9	0	0
	4	7	1	2	8	6	2	7	7	7	1	4	4	6	3	0	0
	7	6	8	5	0	3	9	1	3	1	2	1	5	9	7	1	9
Ch. rufifacies (AY909055)	g	a	t	t	a	c	t	t	c	a	t	t	g	t	c	g	c
Ch. rufifacies (AF083658)	a	g	c	c	g	t	a	c	t	t	c	c	a	c	t	a	t

Table 4. All DNA substitution sites that varied between local and foreign *Chrysomya megacephala* [AY909052 and AF295551]. Nucleotide position numbers correspond to the investigated regions in this study

Species (Accession number)	Nucleotide Position			
	3 5 5	6 6 3	6 9 3	7 9 2
Ch. megacephala (AY909052)	g	c	c	g
Ch. megacephala (AF295551)	c	t	t	a

Table 5. Maximum intraspecific sequences variation obtained for local *Chrysomya megacephala* and *Chrysomya rufifacies*

Gene/region	*Ch. megacephala*		*Ch. rufifacies*	
	Nucleotide Variation	Percentage (%)	Nucleotide Variation	Percentage (%)
COI	5/1533	0.33	4/1533	0.26
COII	2/692	0.29	1/692	0.14
COI + COII	6/2303	0.26	4/2303	0.17
348bp COI fragment	1/348	0.28	2/348	0.57

Table 6. Maximum intraspecific sequence variation between local and foreign species for *Chrysomya megacephala* [AY909052 and AF295551] and *Chrysomya rufifacies* [AY909055 and AF083658]

Species	Nucleotide Variation	Percentage (%)
Ch. megacephala	4/2303	0.17
Ch. rufifacies	17/2303	0.74

Table 7. Minimum interspecific variation for *Chrysomya megacephala* (specimen Fly) and *Chrysomya rufifacies* (specimen CR3)

Gene/region	Nucleotide Variation	Percentage (%)
COI	100/1533	6.12
COII	39/692	5.64
COI + COII	141/2303	6.12
348bp COI fragment	25/348	7.18

calculated for COI and COII regions as well as combined COI+COII, and the 348bp fragment in COI are shown in Table 7. The low variation observed is again expected, as previous studies have shown that intraspecific sequence divergence rarely exceeds 1% (Wells & Sperling, 1999; Harvey *et al.*, 2003b). The same trends were also observed when local *Ch. megacephala* sequences were compared to published foreign *Ch. megacephala* sequences where a maximum of 0.17% divergence was observed (Table 7). Comparison of local and foreign *Ch. rufifacies* sequences showed a higher maximum intraspecific divergence of 0.74%, but notably still within the 1% limit.

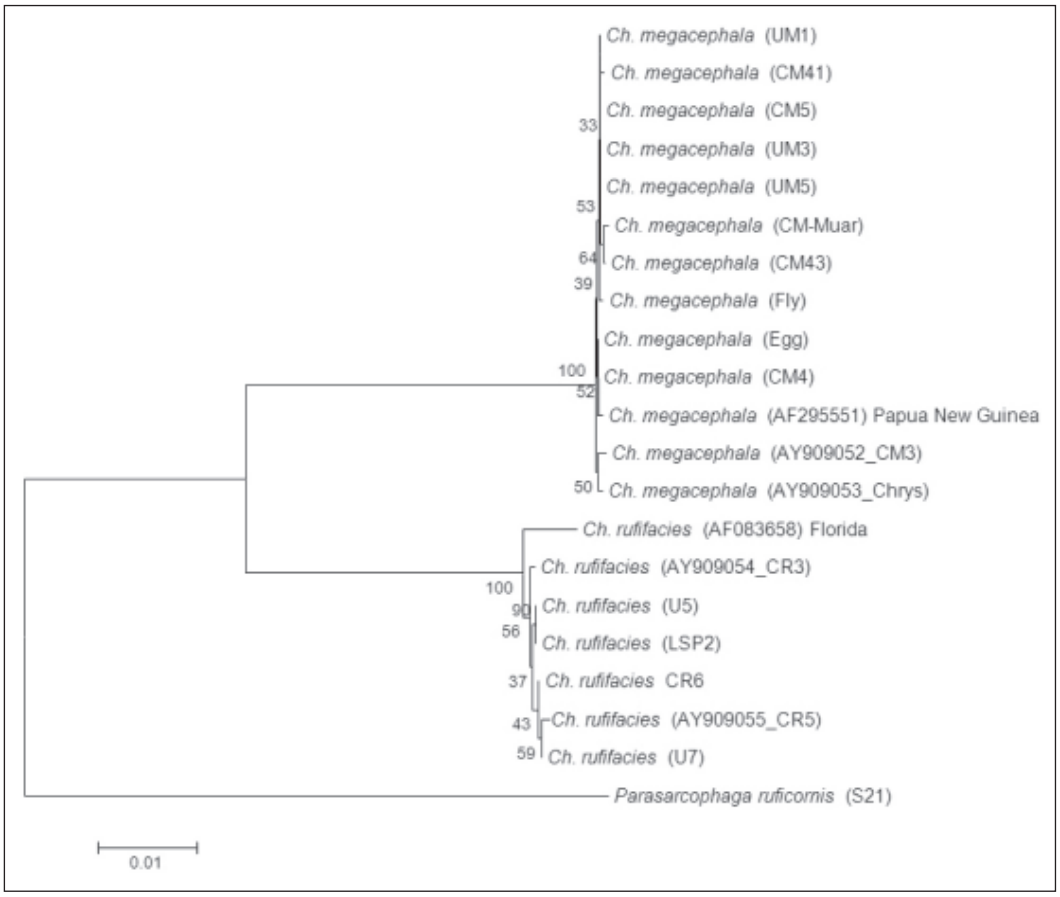

Figure 3. Neighbour-joining tree constructed using MEGA showing phylogenetic relationships of all haplotypes. Numbers above internodes are values of bootstrap support.

When interspecific variation was considered however, marked differences were observed, and thus emphasize the usefulness of this region for species identification purposes (Table 7). It is noteworthy that variations within the 348bp COI fragment showed the highest level of divergence in *Ch. rufifacies*, corroborating the fact that between *Ch. megacephala* and *Ch. rufifacies* sequence information within this region would suffice to separate these two species (Schroeder *et al.*, 2003). A neighbour-joining phylogenetic tree presented in Figure 3 graphically illustrates the distinction of 2.3 kb of COI and COII DNA sequence between these two species as evidenced by the formation of two distinct clusters. The neighbour-joining phylogenetic trees constructed based on COI and COII sequences separately showed similar clusterings. In addition, the use of UPGMA and maximum parsimony methods of phylogenetic calculations also produced similar tree topologies (data not shown).

Results presented in this study are comparable to other published observations from various countries (Stevens & Wall, 2001; Wells & Sperling, 2001). As such, these approaches should be further developed to include more species, especially those that are unique or specifically endemic, as the information would be beneficial not only for forensic analysis but towards the appreciation of Malaysia's biodiversity as a whole. Molecular analysis is able to complement morphology-based identification, and especially particularly useful as an additional source of reference to resolve taxonomical conflicts.

Acknowledgements. Special thanks to K. Ramakrishnan from Department of Parasitology, Faculty of Medicine, University of Malaya for his valuable help in species identification for larval specimens. This study was supported by the Ministry of Science, Technology and Innovation, Malaysia in the forms of National Science Fellowship and short term research attachment programme to the first author. This study was also supported by the short-term grants F0163/2004A, F0181/2005C and PS085/2007B from University of Malaya, Malaysia and the National e-Science Fund 02-01-03-SF0092 received from the Ministry of Science, Technology and Innovation, Malaysia.

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