Appendix

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Mitochondrial DNA-based identification of some forensically important blowflies in Thailand

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ABSTRACT

Accurate identification of insects collected from death scenes provides not only specific developmental data assisting forensic entomologists to determine the postmortem interval more precisely but also other kinds of forensic evidence. However, morphological identification can be complicated due to the similarity among species, especially in the early larval stages. To simplify and make the species identification more practical and reliable, DNA-based identification is preferentially considered. In this study, we demonstrate the application of partial mitochondrial cytochrome oxidase I (COI) and cytochrome oxidase II (COII) sequences for differentiation of forensically important blowflies in Thailand; *Chrysomya megacephala*, *Chrysomya rufifacies* and *Lucilia cuprina* by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The PCR yields a single 1324 bp-sized amplicon in all blowfly specimens, followed by direct DNA sequencing. Taq$^{\alpha}$I and VspI predicted from the sequencing data provide different RFLP profiles among these three species. Sequence analysis reveals no significant intraspecific divergence in blowfly specimens captured from different geographical regions in Thailand. Accordingly, neighbor-joining tree using Kimura's 2-parameter model illustrates reciprocal monophyly between species. Thus, these approaches serve as promising tools for molecular identification of these three common forensically important blowfly species in Thailand.

1. Introduction

Estimation of postmortem interval (PMI) is crucial for time of death determination. Some physiological changes such as body cooling, lividity, and mechanical excitability of skeletal muscle have been used to calculate the early postmortem period. However, such postmortem changes may not be a reliable parameter for approximation of PMI after 48–72 h. After decomposition is initiated, developmental period from hatching to larval stage of insects collected from corpses is the best evidence for PMI estimation [1].

Blowflies play a role in the early decomposition process because of their great abundance and behaviours including feeding and egg laying on corpses. Unsurprisingly, blowfly larvae are usually collected from death scenes and also determined as the best entomological indicator for PMI estimation. The challenging step for forensic entomologists is larval species identification because many morphological similarities among closely related species make difficulties in definite differentiation. A taxonomic key covering all immature stages of common forensically important insect species in all geographical regions is yet unavailable. Although electron microscopy-based identification of some chrysomyine larval stages has been proposed [2], it is not practical and requires many special skills for sample preparation. Alternatively, rearing larvae to the adult stage followed by traditional identification based on the adult morphological characteristics can be performed, but rearing is a time-consuming procedure. Moreover, specimens may be killed or damaged before arrival at the laboratory.

Several studies using DNA-based identification of some forensically important blowfly specimens have been reported [3–7]. These molecular tools can overcome many difficulties associated with morphological problems as previously described. At present, mitochondrial DNA (mtDNA) is preferably applied for

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forensic investigations because greater abundance in tissues, when compared with nuclear DNA (nuDNA), makes it easier for extraction even from small amount of sample [6]. In addition, because of its strictly maternal inheritance and no genetic recombination, mtDNA haplotype is a good candidate for evolutionary and population genetics study. Especially, mitochondrial cytochrome oxidase I and II (COI–COII) genes are suitable as molecular markers because relatively a high degree of genetic variation in this region has been reported [6,7].

Chrysomya megacephala and *Chrysomya rufifacies* are the most common blowfly species in all locations of Thailand and have been extensively documented from forensic cases in both urban and forested areas [8]. *Lucilia cuprina* is another species of forensic interest since it can be found in both urban and forested locations, although less frequently than *Chrysomya* species. Interestingly, it has also been reported that this blowfly species can be found with *C. megacephala* and *C. rufifacies* in the same cadavers [8]. It should be mentioned, however, that molecular identification and genetic relationships of these three blowfly species of forensic importance have not been reported before. Therefore, our approach is to demonstrate the utility of mitochondrial cytochrome oxidase genes for identification purpose and phylogenetic analysis of these three common forensically important blowflies.

2. Materials and methods

2.1. Specimens collection

Adult blowfly specimens were collected from various regions of Thailand including Bangkok, Phitsanulok, Chiang-Mai, Tak, Chumphon and Buri-Ram (Fig. 1) using fly-trap method as previously described [9]. Briefly, pork viscera were used as baits for trapping blowflies in a large plastic bag for 24 h. Flies were anesthesized in a freezer at −20 °C for 1 h and then identified morphologically under a stereomicroscope (SZX9, Olympus, Tokyo, Japan), by reference to the taxonomic key of Crosskey and Lane [10]. The identified flies were stored in 70% ethanol and kept at 4 °C until use.

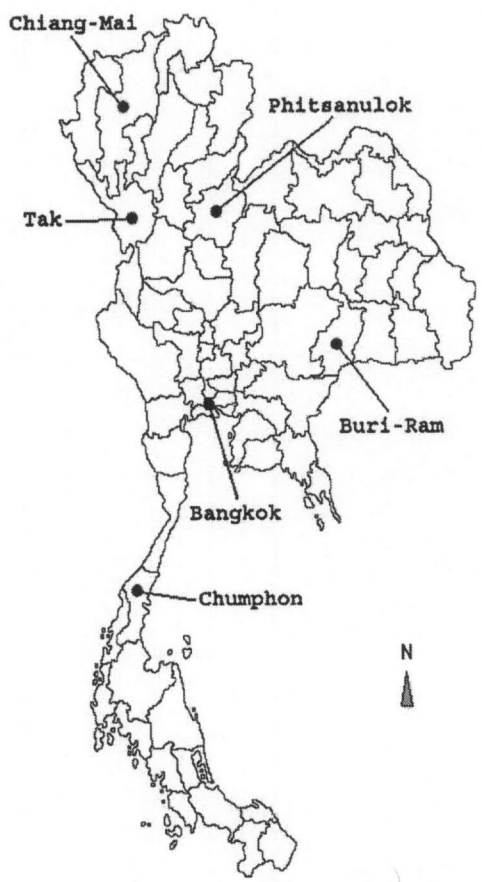

Fig. 1. Map of Thailand, showing the locations for collection of blowfly specimens.

2.2. DNA extraction

Total DNA was prepared from thorax and legs of the fly specimens using QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. The extracted fly DNA was eluted in 200 μl of elution buffer and kept at −20 °C for long term storage. The fraction of extracted DNA was spectrophotometrically quantitated and diluted to 50 ng/μl prior to PCR amplification step.

2.3. PCR amplification

Using the total DNA as a template, partial COI–COII region was amplified. Two oligonucleotide primers including forward primer (5′-CAGCTACTTTATGAGCTTTAGG-3′) and reverse primer (5′-GAGACCATTACTTGCTTTCAGTCATCT-3′) were designed following previous studies [3,7]. The amplification reaction was set up in a final volume of 25 μl, containing 150 ng of extracted DNA, 0.4 μM of each primer, 2.5 mM of $MgCl_2$, 200 μM of dNTPs and 1 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad CA, USA). The PCR reactions were performed in a GeneAmp PCR System 2400 thermal cycler (Applied Biosystem, Foster city, CA, USA) using the condition as follows: the initial denaturation (94 °C for 3 min); the subsequent 5 cycles consisted of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 1.5 min; followed by 25 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1.5 min; and the final extension at 72 °C for 10 min. Aliquots of the amplicons were detected on 1% agarose gel electrophoresis.

2.4. DNA sequencing and restriction patterns prediction

In order to verify unique restriction sites for PCR-RFLP, the PCR products were purified from agarose gel by using a Perfectprep® Gel Cleanup kit (Eppendorf, Germany) following the manufacturer's instructions. Direct DNA sequencing was performed by using ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Applied Biosystems Division, Foster City, CA). Determination of the nucleotide sequences was performed and analyzed in both directions using the same forward and reverse primers used in PCR step to ensure that variations of nucleotide sequences were not due to sequencing errors. The reaction products were analyzed with an automated ABI PRISM® 310 Genetic Analyzer (PerkinElmer, Foster City, CA). The resulting sequences were used for prediction of species-specific restriction sites by using the NEBcutter V2.0 web-based program (available at http://tools.neb.com/NEBcutter2/index.php). From restriction prediction data, two restriction endonucleases (Taq^{α}I and VspI) were chosen for restriction fragment length polymorphism (RFLP).

2.5. RFLP

The PCR products were digested in separate reaction with Taq^{α}I and VspI (New England Biolabs, Ipswich, USA). Reaction mixture was composed of approximate 500 ng of PCR product, 1 μl of 10× appropriate buffer, 2 units of restriction enzyme and DNase-free water to final volume of 10 μl. The mixture was incubated overnight at 65 °C and 37 °C for Taq^{α}I and VspI, respectively. The restriction products were electrophoresed through 6% native polyacrylamide gel electrophoresis 6% gel concentration (T), 3.3% crosslinking (C), 1× TBE buffer (89 mM Tris–base, 89 mM Boric acid, 2 mM EDTA, pH 8.3), run at 100 V for 60 min [11], followed by ethidium bromide staining and visualized on a Gel Doc EQ system (Bio-Rad, CA, USA).

2.6. Sequence analysis and phylogenetic tree construction

Nucleotide sequences were prepared and analyzed using Chromas Lite version 2.01 (http://www.technelysium.com.au) and BLAST search (http://www.ncbi.nlm.gov/BLAST) for species identification. All partial nucleotide sequences of COI–COII genes obtained from this study were submitted to the GenBank database and assigned accession numbers as FJ153258–FJ153278. Sequences were aligned by using the Clustal W algorithm implemented in the BioEdit Sequence Alignment Editor v. 6.0.7 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Percentage of G+C contents and sequence identity matrix were also calculated using the BioEdit program. Phylogenetic tree based on the COI–COII sequences was constructed by neighbor-joining method using the Kimura's 2-parmeter model implemented in the MEGA® version 3.1 [12] and the tree was tested by 1000 bootstrap replicates. Bootstrapping values indicate percentage support for grouping by random resampling of the data.

3. Results

3.1. Amplification of mitochondrial COI–COII genes

Result obtained from PCR amplification and agarose gel electrophoresis revealed that total DNA extracted from thorax and legs of an adult fly was appropriate enough to serve as a template for amplification of mitochondrial COI–COII genes. The specific primers used in this study yielded the PCR-amplified

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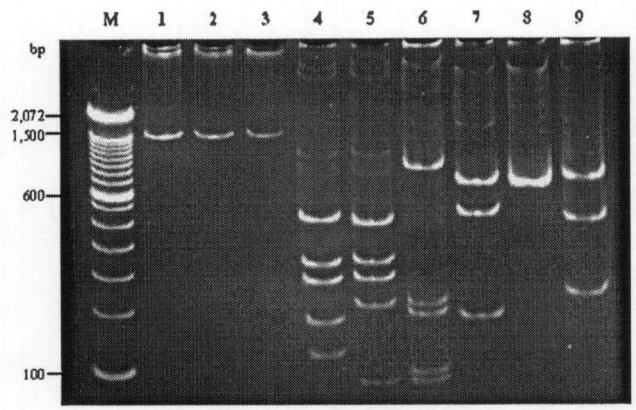

Fig. 2. The 6% native polyacrylamide gel shows different PCR-RFLP patterns of COI–COII amplicons digested with two restriction endonucleases. From left to right as follows: undigested PCR products from *C. megacephala*, *C. rufifacies* and *L. cuprina*, respectively (lanes 1–3); *C. megacephala*/Taq$^{\alpha}$I (lane 4); *C. rufifacies*/Taq$^{\alpha}$I (lane 5); *L. cuprina*/Taq$^{\alpha}$I (lane 6); *C. megacephala*/VspI (lane 7); *C. rufifacies*/VspI (lane 8); *L. cuprina*/VspI (lane 9). Lane M is 100 bp DNA standard marker.

product of 1.3 kbp in length for all blowfly species tested as shown in Fig. 2.

3.2. Identification and distribution of blowfly species in Thailand

In this study, 20 blowflies were collected from 6 different regions of Thailand (Fig. 1) including Bangkok (Center), Chiang-Mai, Tak, Phitsanulok (Northern), Buri-Ram (Eastern) and Chumphon (Southern). Based on the sequence analysis and BLAST search of partial COI–COII sequences (1200 bp in length), the blowflies could be classified into 3 species: *C. megacephala*, *C. rufifacies* and *L. cuprina*. These bioinformatics results were consistent with the taxonomic key-based identification (data not shown). From the 20 blowflies collected, 12 specimens (from all 6 regions of Thailand) were identified as *C. megacephala*; 5 specimens were of *C. rufifacies* (isolated from Bangkok, Chiang-Mai and Phitsanulok), whereas the remaining 3 specimens (collected only in Chiang-Mai) were classified as *L. cuprina* (Table 1).

Table 1
Isolate code, sex, location and accession number of each blowfly used in this study.

Blowfly species	Sex	Location	Accession no.
Chrysomya megacephala			
A01	♂	Tak	FJ153258
A02	♀	Chiang-Mai	FJ153259
A03	♂	Chiang-Mai	FJ153260
A04	♂	Chiang-Mai	FJ153261
A05	♂	Chiang-Mai	FJ153262
A06	♀	Buri-Ram	FJ153263
A07	♀	Buri-Ram	FJ153264
A08	♀	Phitsanulok	FJ153265
A09	♂	Phitsanulok	FJ153266
A10	♂	Chumphon	FJ153267
A11	♂	Chumphon	FJ153268
A12	♀	Bangkok	FJ153269
Chrysomya rufifacies			
B01	♀	Bangkok	FJ153270
B02	♂	Bangkok	FJ153271
B03	♀	Phitsanulok	FJ153272
B04	♀	Chiang-Mai	FJ153273
B05	♂	Chiang-Mai	FJ153274
Lucilia cuprina			
C01	♀	Chiang-Mai	FJ153275
C02	♀	Chiang-Mai	FJ153276
C03	♂	Chiang-Mai	FJ153277
Musca domestica (Outgroup)			
D01	♂	Bangkok	FJ153278

3.3. Discrimination of blowfly species in Thailand based on PCR-RFLP

According to the sequence analysis of partial COI–COII genes, the restriction sites and suitable restriction enzymes were selected by using web-based bioinformatics program (NEBcutter V2.0). Two restriction enzymes (Taq$^{\alpha}$I and VspI) were used in separate reactions in order to generate distinct RFLP patterns and thus be applied for discrimination among different blowfly species. Digestion with Taq$^{\alpha}$I and VspI yielded different RFLP patterns in each species as summarized in Table 2. The representative of restriction patterns for each species is shown in Fig. 2.

3.4. Sequences analysis and comparison

Multiple-alignments of 20 partial mitochondrial COI–COII sequences were performed by using Clustal W implemented in the BioEdit Sequence Alignment Editor version 6.0.7. Analysis of the mean G+C contents revealed slight differences between species, ranging from 28.95% (*C. megacephala*), 28.26% (*L. cuprina*) and 27.37% (*C. rufifacies*). Sequence comparisons between the same species showed that percentage of sequence similarity ranged from 99.7 to 100 (mean 99.91%), 99.6 to 100 (mean 99.72%) and 99.7 to 99.9 (mean 99.80%) for *C. megacephala*, *C. rufifacies* and *L. cuprina*, respectively. The result indicated that there was no significant divergence of partial mitochondrial COI–COII sequences within the same species isolated from different geographical regions in Thailand. On the other hand, sequence comparisons among different blowfly species revealed that percentage of sequence identity between *C. megacephala* and *C. rufifacies* ranged from 93.0 to 93.3 (mean 93.18%), whereas similarity between *C. megacephala* and *L. cuprina* ranged from 60.0 to 60.2 (mean 60.04%) and that between *C. rufifacies* and *L. cuprina* ranged from 60.0 to 60.3 (mean 60.13%). The interspecific sequence comparison indicated that the partial mitochondrial COI–COII sequence provided effective data for discrimination among these three blowfly species in Thailand. Table 3 shows the identity matrix representing the similarity between each individual sequences.

3.5. Phylogenetic analysis

Phylogenetic tree based on COI–COII nucleotide sequences of blowflies from several worldwide geographical areas including available data from GenBank was constructed by neighbor-joining (NJ) method with the Kimura's 2-parmeter model implemented in the MEGA© version 3.1 and the tree were tested by 1000 bootstrap replicates (Fig. 3). *Musca domestica* was used as the outgroup of the phylogenetic tree. According to the tree, all of the *C. megacephala* isolates clustered together showing no significant differentiation between different regions. *C. megacephala* and *C. rufifacies* could be well separated although they belonged to the same genus, implying that the COI–COII sequence was useful for identification of these congeneric species. All of the *C. rufifacies* isolates formed a single cluster with branches indicating minor nucleotide variations between the same species. *L. cuprina* was clearly separated from *C. megacephala* and *C. rufifacies* and all 3 isolates were clustered together.

Table 2
Comparative RFLP patterns among three blowfly species.

Blowfly species	Taq$^{\alpha}$I digestion	VspI digestion
Chrysomya megacephala	30, 111, 156, 264, 307 and 456 bp	4, 12, 160, 495 and 653 bp
Chrysomya rufifacies	30, 81, 186, 264, 307 and 456 bp	12, 653 and 659 bp
Lucilia cuprina	30, 81, 86, 177, 188 and 762 bp	210, 450 and 664 bp

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Table 3
Identity matrix reveals the percentage of partial COI–COII sequence similarity between each isolate.

Code	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B01	B02	B03	B04	B05	C01	C02	C03
A01																				
A02	99.8																			
A03	99.8	100																		
A04	99.8	100	100																	
A05	99.8	100	100	100																
A06	99.8	100	100	100	100															
A07	99.8	100	100	100	100	100														
A08	99.8	100	100	100	100	100	100													
A09	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9												
A10	99.8	100	100	100	100	100	100	100	99.9											
A11	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.9										
A12	99.7	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.8	99.9	99.8									
B01	93.1	93.3	93.3	93.3	93.3	93.3	93.3	93.3	93.2	93.3	93.2	93.2								
B02	93.1	93.3	93.3	93.3	93.3	93.3	93.3	93.3	93.2	93.3	93.2	93.2	99.8							
B03	93	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93	93.1	93	93	99.6	99.6						
B04	93.1	93.3	93.3	93.3	93.3	93.3	93.3	93.3	93.2	93.3	93.2	93.2	99.8	100	99.6					
B05	93	93.1	93.1	93.1	93.1	93.1	93.1	93.1	93	93.1	93	93	99.6	99.8	99.6	99.8				
C01	60.1	60	60	60	60	60	60	60	60	60	60.1	60	60	60.1	60.2	60.1	60.1			
C02	60.2	60.1	60.1	60.1	60.1	60.1	60.1	60.1	60	60.1	60.2	60	60.1	60.2	60.3	60.2	60.2	99.9		
C03	60.1	60	60	60	60	60	60	60	60	60	60.1	60	60	60.1	60.2	60.1	60.1	99.7	99.8	
D01	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	57.4	58.4	58.4	58.3	58.4	58.3	53.3	53.2	53.4

A01–A12, *Chrysomya megacephala*; B01–B05, *Chrysomya rufifacies*; C01–C03, *Lucilia cuprina*; D01, *Musca domestica*.

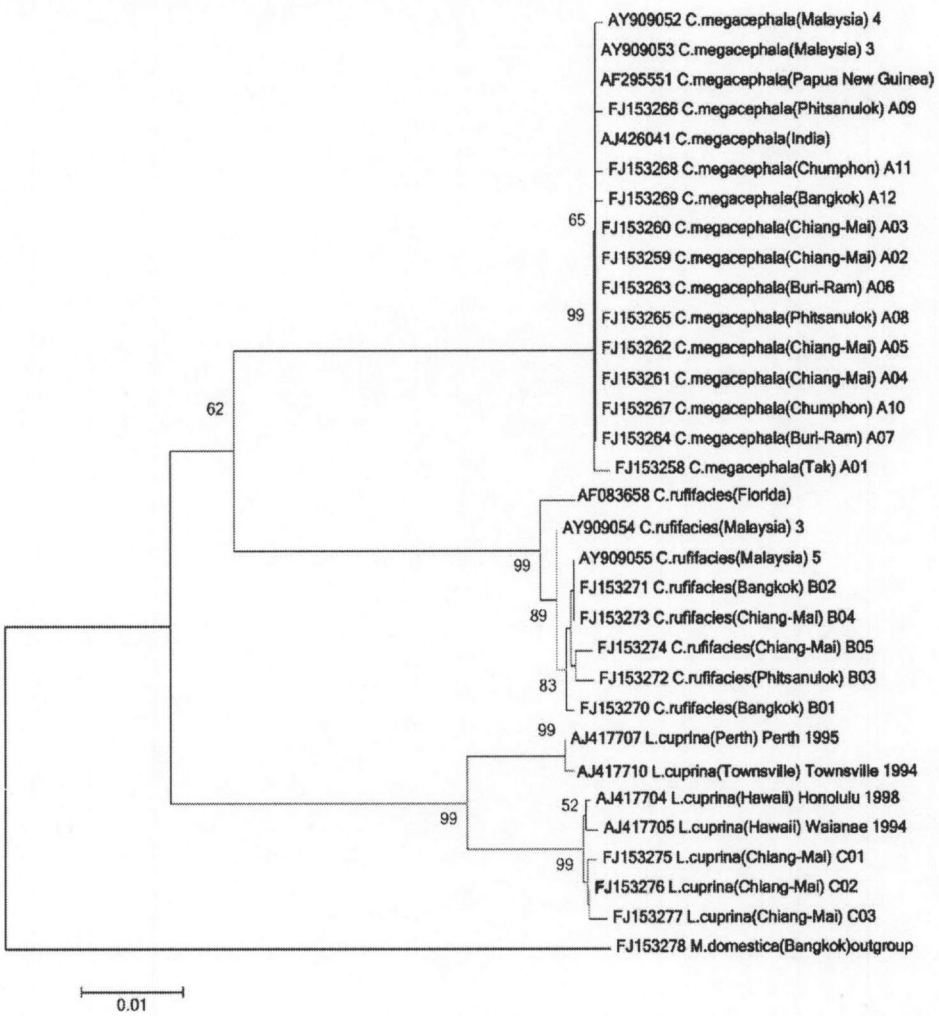

Fig. 3. The neighbor-joining tree using Kimura's 2-parameter model illustrating phylogenetic relationships among three blowfly species and one housefly outgroup, based on the COI–COII nucleotide sequences data.

4. Discussion

In accordance with previous reports [13–15], *C. megacephala* is the most common blowfly species and more easily captured than other species, throughout Thailand. Boonchu et al. [9] found that *C. megacephala* (96.3%) represented the majority of all species whereas *C. rufifacies* (0.2%) was rarely captured by this trapping method. A possible explanation for their abundance is their capacity to utilize a wide range of ecological niches throughout Thailand. Additionally, each blowfly species may be differentially

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attracted by the baits used. Sukontason et al. [8] reported that only *L. cuprina*, not *L. sericata*, was found in forensic cases in Thailand and, to our knowledge, *L. sericata*, the sister species of *L. cuprina*, has never yet been reported in forensic entomology cases in Thailand.

Although PCR-RFLP used to be a preferred method for polymorphism demonstration, this method has some problems since restriction sites can mutate or get lost despite no morphological change. In addition, some authors state that identification by this strategy will be accurate when enough samples of overall flies in the taxa are done as reference profiles [16,17]. However, RFLP serves some advantages for forensic personnel, by partly assisting in the exclusion of some fly species and for screening purposes.

In this study, we demonstrated the value of PCR-RFLP in differentiating three blowfly species, *C. megacephala*, *C. rufifacies* and *L. cuprina*. The 6% native polyacrylamide gel showed good resolution for discriminating two small fragments (81 and 86 bp) for *L. cuprina* by Taq$^{\alpha}$I digestion. On the other hand, VspI digestion provided two larger fragments (653 and 659 bp) overlapping like a single band on the gel, for *C. rufifacies*. VspI also demonstrated different profiles between *C. megacephala* and *L. cuprina*. Very small fragments (<50 bp) often disappear in Fig. 2 due to low ethidium bromide fluorescence intensity and possible diffusion during electrophoresis. All samples after digestion by Taq$^{\alpha}$I and VspI showed similar restriction profiles within same species (data not shown). This result showed that intraspecific polymorphism was not observed here by RFLP. Ethidium bromide-stained polyacrylamide gel techniques demonstrate that the different species-specific restriction profiles are suitable to apply in routine forensic laboratory tests. Although the PCR-RFLP can differentiate these three blowfly species, fly specimens collected from the death scene could be of other closely related species [16–18], a situation which could potentially lead to misidentification errors.

To distinguish sister species more effectively, phylogenetic analysis has been widely employed [16–19]. From Fig. 3, maximum parsimony phylogeny constructed from global data (available in GenBank) reveals reciprocal monophyly between species. Table 3 also shows that intraspecific variation was just detected ≤0.3% for *C. megacephala*, ≤0.4% for *C. rufifacies* and ≤0.3% for *L. cuprina*, supporting monophyletic pattern. However, Wells et al. [16–18] have emphasized the importance of using a broad enough genetic database of all relevant species as an essential key for accurate species identification by phylogenetic analysis of COI sequence. In addition, phylogenies based on different test loci may be discordant. Stevens et al. [19] found that phylogenies constructed from worldwide *L. cuprina* and *L. sericata* were paraphyletic using COI but each monophyletic for 28S ribosomal RNA gene. Therefore, the pitfalls of this study are small sample size and lack of genetic database of close relatives at different test loci for validating the methods. However, the current study successfully demonstrates not only the application of mitochondrial cytochrome oxidase genes for species identification, but also provides phylogenetic information for these common forensically important blowflies from several geographical areas of Thailand.

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