

Use of Cytochrome c Oxidase Subunit I (*COI*) Nucleotide Sequences for Identification of the Korean Luciliinae Fly Species (Diptera: Calliphoridae) in Forensic Investigations

Blowflies, especially species belonging to the subfamily Luciliinae, are the first insects to lay eggs on corpses in Korea. Fast and accurate species identification has been a key task for forensic entomologists. Because conventional morphologic identification methods have many limitations with respect to forensic practice, molecular methods have been proposed to identify fly species of forensic importance. To this end, the authors amplified and sequenced the full length of the cytochrome c oxidase subunit I (*COI*) gene of the Luciliinae fly species collected in Korea. The results showed the *COI* sequences are instrumental in identifying Luciliinae fly species. However, when compared with previously reported data, considerable inconsistencies were noted. *Hemipyrellia ligurriens* data in this study differed significantly from two of the five pre-existing data. Two closely related species, *Lucilia illustris* and *Lucilia caesar*, showed an overlap of *COI* haplotypes due to four European sequences. The results suggest that more individuals from various geographic regions and additive nuclear DNA markers should be analyzed, and morphologic identification keys must be reconfirmed to overcome these inconsistencies.

Key Words : Forensic Medicine; Entomology; Diptera; Identification; Calliphoridae; Cytochrome-c Oxidase

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INTRODUCTION

Estimation of the postmortem interval (PMI) is an essential component in investigations of unnatural death (1). Before a corpse begins to decompose, the PMI can be estimated by observation of early postmortem changes, such as body cooling, muscle rigidity, and postmortem lividity. These early postmortem changes are the results of physical or chemical consequences, which do not alter the overall consistency of the corpse. However, because the early postmortem changes are disrupted by late postmortem changes, such as putrefaction and autolysis, which gradually degrade the corpse, estimation of the PMI in a decomposing body has been a difficult task in forensic practice (1). Tiny insects gathering around the corpse can provide an alternative clue because the growth rates and the occurrence of insects are dependent on the ambient temperature and the stages of decomposition, respectively (2). In most developed countries, forensic entomology, which applies insect data to the forensic investigation, is now a major approach used to estimate PMI after decomposition occurs.

Because different insect species have different growth rates

and different geographic or climatic distributions, accurate species identification is critical in the practice of forensic entomology (3-6). However, species identification of insects of forensic importance, especially flies, is a very complicated and difficult process, in which the small anatomic identification keys are observed with a stereomicroscope (7). 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 (8). For accurate and convenient species identification, DNA-based methods have been developed, usually analyzing the mitochondrial cytochrome c oxidase I (*COI*) gene or the ribosomal internal transcribed spacer 2 (*ITS 2*) (9-11). Because DNA sequences are divergent among different species, species identification is possible with the DNA sequence data. Currently, *COI* gene sequences are used as a main molecular identification tool for insects (12-14).

The subfamily Luciliinae is one of the major groups in the Family Calliphoridae (7). However, there have been limited studies about *COI* sequences of Korean Luciliinae fly species (15, 16). Furthermore, full-length sequences of *COI* genes

of the Korean Luciliinae fly species have not yet been determined. The current study is the first to determine the full-length sequences of *COI* genes of Korean Luciliinae flies, which the authors believe, are the most important necrophagous fly species, from late spring to early fall, in Korea.

MATERIALS AND METHODS

Fly collection and species

Flies were collected from various locations in Seoul, Korea. Morphologic identification was performed under a dissecting

Table 1. *COI* sequences used in this study

GenBank accession number	Species name	Sequence coverage on <i>COI</i>	Length (Nucleotide)	Location	Reference
EU880193-EU880196	<i>L. caesar</i>	All	1,539	Korea	Newly sequenced
EU880197-EU880205	<i>L. illustris</i>	All	1,539	Korea	Newly sequenced
EU880206-EU880207	<i>H. ligurriens</i>	All	1,539	Korea	Newly sequenced
EU880208-EU880212	<i>P. sericata</i>	All	1,539	Korea	Newly sequenced
EU925394	<i>L. ampullacea</i>	All	1,539	Korea	Newly sequenced
AY097334	<i>H. ligurriens</i>	All	1,539	Taiwan	Chen et al. (17)
AY842614	<i>H. ligurriens</i>	717-1,536	820	Australia	Wallman et al. (18)
DQ345092	<i>H. ligurriens</i>	220-1,533	1,314	China	Zhu et al.
DQ453493	<i>H. ligurriens</i>	All	1,539	Taiwan	Wells et al. (19)
EU047576	<i>H. ligurriens</i>	717-995	279	Malaysia	Mahat et al.
EU661350	<i>H. ligurriens</i>	256-732	477	China	Yang et al.
DQ453487	<i>L. ampullacea</i>	All	1,539	U.K.	Wells et al. (19)
EU418575	<i>L. ampullacea</i>	303-1,469	1,167	France	Harvey et al. (20)
AJ417703	<i>L. caesar</i>	All	1,539	U.K.	Stevens et al. (21)
AY092781	<i>L. caesar</i>	220-741	522	U.K.	Pickles et al.
AY092782	<i>L. caesar</i>	220-741	522	U.K.	Pickles et al.
AY092783	<i>L. caesar</i>	220-741	522	U.K.	Pickles et al.
AY092784	<i>L. caesar</i>	220-741	522	U.K.	Pickles et al.
AY092785	<i>L. caesar</i>	220-741	522	U.K.	Pickles et al.
DQ453488	<i>L. caesar</i>	All	1,539	U.K.	Wells et al. (19)
FJ025632	<i>L. caesar</i>	30-635	1,271	Singapore	Kutty et al.
		791-1,455			
AJ551445	<i>L. illustris</i>	All	1,539	U.K.	Stevens (22)
AY092791	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
AY092792	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
AY092793	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
AY092794	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
AY092795	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
AY092796	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
AY092797	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
AY092798	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
AY092799	<i>L. illustris</i>	220-741	522	U.K.	Pickles et al.
DQ345090	<i>L. illustris</i>	220-1,533	1,314	China	Zhu et al.
EU418574	<i>L. illustris</i>	303-1,469	1,167	France	Harvey et al. (20)
L14945	<i>L. illustris</i>	All	1,539	Unknown	Sperling et al. (9)
AJ417704	<i>P. cuprina</i>	All	1,539	Hawaii	Stevens et al. (21)
AJ417705	<i>P. cuprina</i>	All	1,539	Hawaii	Stevens et al. (21)
DQ453495	<i>P. cuprina</i>	1-1,536	1,536	Hawaii	Wells et al. (19)
DQ453496	<i>P. cuprina</i>	1-1,536	1,536	Hawaii	Wells et al. (19)
AJ417712	<i>P. sericata</i>	All	1,539	Denmark	Stevens et al. (21)
AJ417713	<i>P. sericata</i>	All	1,539	New Zealand	Stevens et al. (21)
AJ417714	<i>P. sericata</i>	All	1,539	U.K.	Stevens et al. (21)
AJ417715	<i>P. sericata</i>	All	1,539	Australia	Stevens et al. (21)
AJ417716	<i>P. sericata</i>	All	1,539	Spain	Stevens et al. (21)
AJ417717	<i>P. sericata</i>	All	1,539	Zimbabwe	Stevens et al. (21)
L14947	<i>P. sericata</i>	All	1,539	Unknown	Sperling et al. (9)

*The initials H, L, and P represent *Hemipyrellia*, *Lucilia*, and *Phaenicia*, respectively; ¹U.K. refers to the United Kingdom.

microscope by a taxonomic expert in dipterology, following procedures published by Kano and Shinonaga (7). Four individuals of *Lucilia caesar*, nine *Lucilia illustris*, two *Hemipyrellia ligurriens*, five *Phaenicia sericata*, and one *Lucilia ampullacea* were examined (Table 1).

DNA extraction

After the compound eyes were removed, the whole bodies were ground to a fine powder using liquid nitrogen. Four hundred μL of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, and 0.5% SDS [pH 8.0]) and 10 μL of proteinase K (20 $\mu\text{g}/\mu\text{L}$) were added to the powder and the mixture was incubated at 56°C for 3 hr. DNA was extracted from the mixture using a conventional phenol/chloroform/isoamylalcohol method (23).

Primer design

Two new primers, which span the whole *COI* region, were selected from the highly conserved tRNA-cystein and cytochrome c oxidase subunit II (*COII*) regions of the mitochondrial sequences of *Chrysomya putoria* (NCBI accession number NC002697), *Cochliomyia hominivorax* (NC002660), and *Haematobia irritans* (NCBI accession number NC007102). For amplification failures, several internal primers were adopted from other authors' literatures (24-26). The primer sequences are listed in Table 2.

PCR amplification and sequencing

2.5 μL of 10 \times Gold Buffer (Applied Biosystems, Foster City, CA, U.S.A.), 62.5 nM MgCl₂, 5 nM dNTP mix (each), 5 pM primers, and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) were admixed in a 25 μL total volume. After 1 cycle at 95°C for 11 min of hot start enzyme activation, *COI* genes were amplified by 35 cycles at 95°C for 30 sec, annealing temperature for 30 sec, 72°C for 90 sec, and 72°C for 15 min of final extension with GeneAmp 2720 or

9600 PCR Systems (Applied Biosystems). The annealing temperature was optimized for each reaction (55 to 45°C). For sequencing reaction, the residual dNTP and primers in the mixture were processed by incubation at 37°C for 45 min with 10 U of calf intestinal phosphatase and 10 U of exonuclease I (New England BioLabs Inc., Ipswich, MA, U.S.A.). The Big-Dye Terminator, v1.1 or v3.1, Kit (Applied Biosystems) was used for DNA sequencing reaction. Sequencing reaction products were purified following the manufacturer's manual and analyzed by automatic DNA sequencers (ABI PRISM 3730xl genetic analyzer or an ABI PRISM 310 genetic analyzer).

Phylogenetic analysis

After sequence alignment, phylogenetic analysis was performed using the neighbor-joining method with a p-distance model and 1,000 replicates of bootstrapping using MEGA 4 software (27). A *COI* sequence of *Calliphora vicina* (AJ417702) was used as the outgroup. Calculations for percent nucleotide distances were performed using MEGA4 software (27).

Comparison with previously reported sequences

COI nucleotide sequences of Korean Luciliinae flies were aligned with those from other researchers. Pairwise intraspecific percent distances were estimated using MEGA4 software.

RESULTS

COI nucleotide comparison and phylogenetic relationships between Korean Luciliinae fly species

Twenty-one individual flies from three genera and five species in total were examined. The distance matrices of Korean Luciliinae flies are demonstrated in Table 3. The maximum intraspecific distances were 0.2, 0.0, 0.8, and 0.4% for *P. sericata*, *H. ligurriens*, *L. caesar*, and *L. illustris*, respectively. Because only

Table 2. Primer sequences and binding locations

Primers	Sequences	Binding locations
F1	5'-CCTTTAGAATTGCAGTCTAATGTCA-3'	tRNA-Cys
F2	5'-GGAGGATTTGGAATTGATTAGTTCC-3'	220-245 on <i>COI</i>
F3	5'-CAACATTTATTTTGATTCTTTGG-3'	688-710 on <i>COI</i>
F4	5'-CTGCTACTTTATGAGCTTTAGG-3'	1,000-1,022 on <i>COI</i>
R1	5'-CCTAAATTTGCTCATGTTGACA-3'	2-23 on <i>COII</i>
R2	5'-CAAGTTGTGAAGCATC-3'	1,327-1,343 on <i>COI</i>
R3	5'-CCAAAGAATCAAATAAATGTTG-3'	688-710 on <i>COI</i>

Table 3. Percent distance matrices of *COI* nucleotide sequences of Luciliinae flies collected in Korea

	Se	Li	Ca	Il	Am	AJ417702
Se	0.0-0.2					
Li	6.3-6.4	0.0				
Ca	5.2-5.5	6.8-7.3	0.1-0.8			
Il	5.1-5.7	7.2-7.4	1.4-1.9	0.1-0.5		
Am	6.2-6.3	8.1	4.7-4.9	4.8-5.0	-	
AJ417702	7.6	8.7	7.6-7.8	7.3-7.6	8.7	-

*The letters Se, Li, Ca, Il, and Am represent *Phaenicia sericata*, *Hemipyrellia ligurriens*, *Lucilia caesar*, *Lucilia illustris*, and *Lucilia ampullacea*, respectively. AJ417702 is a *COI* sequence of *Calliphora vicina* (outgroup). Numbers in bold indicate intraspecific distances. The intraspecific distance for *Lucilia ampullacea* is not available because there is only one sequence. Sequence lengths are all 1,539.

one individual *L. ampullacea* was available, intraspecific variation in this species was not estimated. Interspecific distance was the lowest between *L. caesar* and *L. illustris* (1.4-1.9%) and the highest between *L. ampullacea* and *H. ligurriens* (8.1%). A phylogenetic tree generated from *COI* sequences of Korean Luciliinae flies showed no paraphyly within the genera or species levels (Fig. 1). Because there was no overlap between interspecific and intraspecific distances, the five Korean Luciliinae fly species examined in this study were distinguishable from each other by comparing *COI* nucleotide sequences.

The number of polymorphic sites was 192 for total data. After intraspecific polymorphic sites were excluded, 155 polymorphic sites remained. These 155 sites are distributed a bit more in the downstream region of the *COI* gene (Fig. 2). This richness of interspecific polymorphism in the downstream region may suggest, in Luciliinae fly species, DNA barcoding, which spans the upstream region, may be not as useful as in other animal taxa. However, the best region choice for species identification may be controversial, because there have been no large-scale studies to investigate the whole length of *COI* genes pertaining to the variable Luciliinae species of forensic importance.

Comparison to previously reported sequences

Table 4 demonstrates intraspecific *COI* nucleotide distances

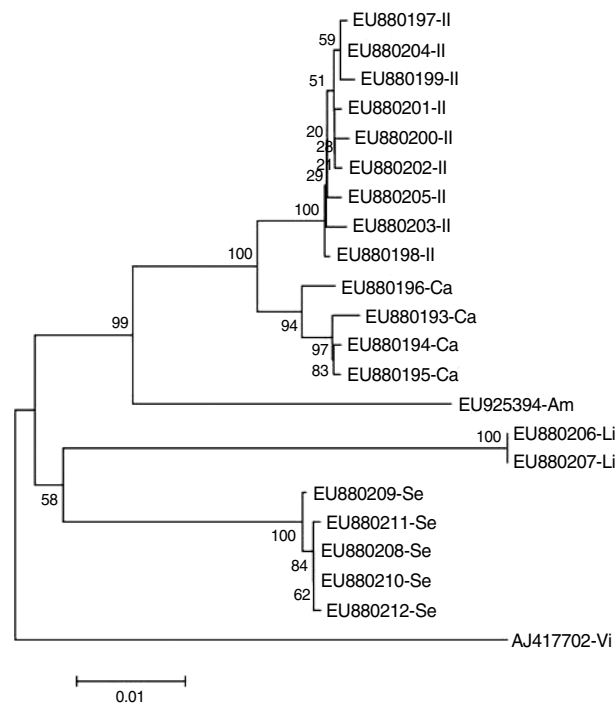


Fig. 1. Phylogenetic analysis of Korean Luciliinae fly species. The letters Se, Am, Ca, Il, Li and Vi stand for *Phaenicia sericata*, *Lucilia ampullacea*, *L. caesar*, *L. illustris*, *Hemipyrellia ligurriens*, and *Calliphora vicina*, respectively. *Calliphora vicina* is included as the outgroup.

from Korean Luciliinae fly species and their previously reported conspecifics. The intraspecific sequence distances among *H. ligurriens* are outstanding (0-8.6%). A separate distance matrix for this species (data not shown) reveals these unusually high intraspecific distances are thoroughly due to two Taiwanese sequences (AY097334 and DQ453493), both morphologically inspected by Chen et al. (17, 19). These two Taiwanese sequences showed 5.5 to 8.6% distances from the conspecifics of other countries. Moreover, these two Taiwanese sequences are unusually similar to those of the Hawaiian *Phaenicia cuprina* (sequence distance 0.4-0.6%), which is a closely related sister species of *P. sericata*. Two closely related sister species, *L. illustris* and *L. caesar* showed 2.8% and 2.5% of intraspecific distances, respectively. These relatively high intraspecific distances are due to four European sequences (two *L. illustris* AJ551445 and EU418574, and two *L. caesar* AJ 417703 and DQ453488). In a phylogenetic analysis, these four sequences caused species-level paraphylies by clustering with their sister species (phylogenetic tree not shown). *P. sericata* and *L. ampullacea* showed 0-0.3% intraspecific distances.

DISCUSSION

Species-level paraphyly or even polyphyly in mitochondrial DNA, is not uncommon in the Animal Kingdom. Accord-

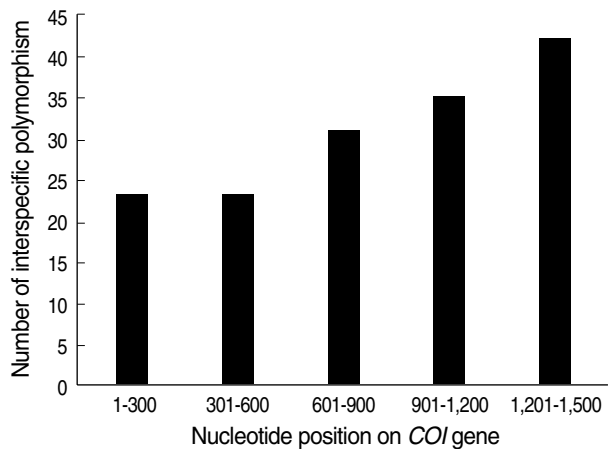


Fig. 2. Distribution of interspecific polymorphic sites on the *COI* gene from site 1 to 1,500.

Table 4. Intraspecific *COI* sequence percent distances between Korean Luciliinae flies and their previously reported conspecifics

Species	Distances (%)
<i>Phaenicia sericata</i>	0-0.3
<i>Lucilia ampullacea</i>	0-0.3
<i>Lucilia illustris</i>	0-2.8
<i>Lucilia caesar</i>	0-2.5
<i>Hemipyrellia ligurriens</i>	0-8.6

ing to Funk and Omland (28), at least 702 arthropod species are polyphyletic (26.5%). For these polyphylics, several explanations have been offered, which consist of inadequate sequence data, inaccurate taxonomy, interspecific hybridization or introgression, and incomplete sorting. In another study by Cognato (29), the intraspecific percent sequence distance of the *COI* genes in a lice species, *Columbicola macrourae*, was the highest (26.0%) among 26 insect species. However, this extraordinary intraspecific percent distance may be due to parasitic inhabitation of the louse (30). According to Cognato (29), intraspecific percent distances of the *COI* genes in 8 fly species (Order: Diptera) range from 0.04 to 3.5%. Therefore, intraspecific percent distances in *H. ligurriens*, 0 to 8.6% (in the Results section), are quite unusual among fly species. Although Wells et al. (19) insisted that validity of the genus *Hemipyrellia* is highly questionable, in our analysis, all sequences of *H. ligurriens*, except for two Taiwanese sequences, show distances within 3.6%. Further investigations, by other taxonomists, into the Taiwanese *H. ligurriens* formerly identified by Chen et al. (17), could ascertain the level of applicability, substantiate findings, and answer questions regarding the unusual *COI* haplotype.

L. illustris and *L. caesar*, which showed overlaps between intra- and interspecific sequence distances, are closely related sister species. Wells et al. (19) also noted a paraphyly between these two species and indicated, because *L. caesar* does not exist in the New World, identification of *L. illustris* in North America would be possible. Despite this paraphyly, because mixed clades occurred only in European strains, Asian strains are possibly distinguishable with *COI* sequence analyses. However, more sequence collection from various Asian and European regions would be helpful to prevent possible misidentification and to reinforce the validity of paraphyletic European sequences. Harvey et al. (20) insisted that an alternative gene choice, perhaps nuclear markers like *ITS* regions, may resolve *COI* para/polyphylics in some Calliphoridae taxa, especially when it is caused by incomplete lineage sorting. Regarding *L. caesar* and *illustris*, alternative gene analyses, including nuclear genes, may be applicable.

In conclusion, the Korean Luciliinae fly species are identifiable with *COI* sequence analyses. However, because of the inconsistent *COI* haplotypes of *H. ligurriens*, *L. caesar*, and *L. illustris* in a worldwide-scale comparison, more individuals from various geographic regions and alternative nuclear genes, for these three species, should be investigated.

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