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TECHNICAL NOTE

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Sequences of the Cytochrome C Oxidase Subunit I (COI) Gene are Suitable for Species Identification of Korean Calliphorinae Flies of Forensic Importance (Diptera: Calliphoridae)

ABSTRACT: Calliphorinae fly species are important indicators of the postmortem interval especially during early spring and late fall in Korea. Although nucleotide sequences of various Calliphorinae fly species are available, there has been no research on the cytochrome c oxidase subunit I (COI) nucleotide sequences of Korean Calliphorinae flies. Here, we report the full-length sequences of the COI gene of four Calliphorinae fly species collected in Korea (five individuals of *Calliphora vicina*, five *Calliphora lata*, four *Triceratopyga calliphoroides* and three *Aldrichina grahami*). Each COI gene was amplified by polymerase chain reaction and directly sequenced and the resulting nucleotide sequences were aligned and analyzed by MEGA4 software. The results indicate that COI nucleotide sequences can be used to distinguish between these four species. Our phylogenetic result coincides with recent taxonomic views on the subfamily Calliphorinae in that the genera *Aldrichina* and *Triceratopyga* are nested within the genus *Calliphora*.

KEYWORDS: forensic science, forensic entomology, postmortem interval, Calliphorinae, Calliphoridae, Diptera, cytochrome c oxidase subunit I, species identification

Identification of fly species is essential in forensic entomology, which provides valuable knowledge for estimating the postmortem interval (PMI) (1). A lack of identification keys for immature stages limits the utilization of conventional morphological identification methods. Therefore, many authors have proposed DNA-based identification methods, especially using the cytochrome c oxidase subunit I (COI) gene (2–6). The earliest visitors to a corpse are usually flies, especially those in the family Calliphoridae (7), which mostly belong to one of three subfamilies: Calliphorinae, Luciliinae, and Chrysomynae. Subfamily Calliphorinae consists of several genera such as *Calliphora*, *Aldrichina*, *Triceratopyga*, *Eucalliphora*, and *Onesia*. Calliphorinae flies in Japan inhabit relatively cooler climates than other Calliphoridae flies do (8–10), which is also confirmed by our observation of Korean Calliphorinae flies (not published). Calliphorinae flies are important indicators of PMI especially during the early spring or late fall in Korea, when flies in the subfamily Luciliinae are not active. Although Calliphorinae flies share many biological characteristics, the duration of immature stages differs considerably among Calliphorinae species (10–13). Therefore, inaccurate species identification may cause errors in estimating PMI. Although many DNA sequences of Calliphorinae species from various geographic regions have been analyzed previously, COI sequences of the Calliphorinae flies in Korea have not yet been reported. Here, we report the full-length sequences (1539 nucleotides) of the COI gene of four forensically important Calliphorinae species: *Calliphora vicina*, *Calliphora lata*, *Triceratopyga calliphoroides*, and *Aldrichina grahami* collected in Korea. To our knowledge, this is the first report of the COI sequence of *T. calliphoroides* and the full sequences of COI genes of *C. lata* and *A. grahami*.

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Materials and Methods

Fly Collection and Species

Adult flies were collected from various regions of South Korea using baits such as pork liver or raw squid. Morphological identification was done under a dissecting microscope by an expert dipterologist with strict criteria (8). Five *C. vicina*, five *C. lata*, four *T. calliphoroides*, and three *A. grahami* were examined in this study (Table 1).

DNA Extraction

Whole bodies, except compound eyes, were dried at 55°C, and ground to powder. DNA was extracted using the conventional phenol/chloroform/isoamylalcohol method (14).

Primer Design

After aligning mitochondrial sequences of *Chrysomya putoria* (NCBI accession number NC002697), *Cochliomyia hominivorax* (NC002660), and *Haematobia irritans* (NC007102), upstream and downstream primers were selected from the tRNA-cysteine and the cytochrome c oxidase subunit I regions, respectively. Several internal primers were selected or modified from published articles for some samples that failed to produce visible DNA bands with the

TABLE 1—*COI sequences used in this study.*

GenBank Accession Number	Species Name	Coverage (length)	Location	Reference
EU880176-EU880179	*Triceratopyga calliphoroides*	1–1,539 (1,539)	South Korea	Newly sequenced
EU880180-EU880182	*Aldrichina grahami*	1–1,539 (1,539)	South Korea	Newly sequenced
EU880183-EU880187	*Calliphora lata*	1–1,539 (1,539)	South Korea	Newly sequenced
EU880188-EU880192	*Calliphora vicina*	1–1,539 (1,539)	South Korea	Newly sequenced
AY818124	*Aldrichina grahami*	1,068–1,345 (278)	Western China	Cai et al. (unpublished)
DQ328667	*Aldrichina grahami*	1,001–1,348 (348)	China	Yin et al. (unpublished)
DQ345097	*Aldrichina grahami*	220–1,533 (1,314)	China	Zhu et al. (unpublished)
DQ345074	*Calliphora augur*	220–1,533 (1,314)	China	Zhu et al. (unpublished)
AF259505	*Cynomya cadaverina*	1–1,539 (1,539)	U.S.A.	Wells (21)
N/A*	*Calliphora lata*	1,023–1,326 (304)	Japan	Saigusa et al. (9)
DQ345093	*Calliphora loewi*	220–1,533 (1,314)	China	Zhu et al. (unpublished)
DQ345094	*Calliphora nigribarbis*	220–1,533 (1,314)	China	Zhu et al. (unpublished)
DQ345095	*Calliphora pattoni*	220–1,533 (1,314)	China	Zhu et al. (unpublished)
AJ417702	*Calliphora vicina*	1–1,539 (1,539)	U Bristol colony, U.K.	Stevens et al. (22)
AY842603	*Calliphora vicina*	719–1,536 (815)	Australia	Wallman et al. (23)
AY842604	*Calliphora vicina*	716–1,532 (815)	Australia	Wallman et al. (23)
DQ345096	*Calliphora vicina*	220–1,533 (1,314)	China	Zhu et al. (unpublished)
AF295557	*Eucalliphora latifrons*	1–1,533 (1,533)	Canada	Wells and Sperling (24)
L14945	*Lucilia illustris*	1–1,539 (1,539)	N/A*	Sperling et al. (2)

*N/A stands for "not available."

initial primer set (9,15,16). Primer sequences used in this study are listed in Table 2.

Polymerase Chain Reaction and Sequencing

Polymerase chain reaction (PCR) was performed with 2.5 μL 10 × Gold Buffer (Applied Biosystems, Foster City, CA), 6.25 nmole $MgCl_2$, 5 nmole (each) dNTP mix, 5 pmole primers and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in the 25 μL reaction volume. The thermal cycle conditions were 1 cycle at 95°C for 11 min, 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 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 temperatures were optimized for each reaction. After PCR, remaining dNTPs and primers in PCR products were inactivated by 10 U calf intestinal phosphatase and 10 U exonuclease I (New England BioLabs Inc., Ipswich, MA). DNA sequencing reactions were performed with the BigDye Terminator v1.1 or v3.1 (Applied Biosystems). DNA sequences were analyzed mainly on an ABI PRISM 3730xl genetic analyzer or partly on an ABI PRISM 310 genetic analyzer.

TABLE 2—*Primer sequences.*

Primer Names	Sequences	Binding Locations
F1	CCTTTAGAATTGCAGTCTAATGTCA	tRNA-Cysteine
F2	GGAGGATTTGGAAATTGATTAGTTCC	220–245 on COI
F3	CAACATTTATTTTGATTCTTTGG	688–710 on COI
F4	CTGCTACTTTATGAGCTTTAGG	1000–1022 on COI
R1	CCTAAATTTGCTCATGTTGACA	2–23 on COII
R2	CAAGTTGTGTAAGCATC	1327–1343 on COI
R3	CCAAAGAATCAAAATAAATGTTG	688–710 on COI

Phylogenetic Analysis

Phylogenetic analysis was performed using MEGA4 software (17). Newly determined nucleotide sequences were compared with previously reported sequences in NCBI GenBank or literatures (Table 1). The phylogenetic tree was generated by neighbor-joining method with a p-distance model and 1000 replicates of bootstrapping. Gaps were considered as "complete deletion." Sequences shorter than 1.3 kb were excluded from the phylogenetic analysis to avoid decrease of number of sites. Shorter sequences were compared by pairwise percent distances. Percent distances were also calculated by MEGA4 software.

Results and Discussion

We analyzed the full-length sequences of COI genes of four Calliphorinae species from three genera. The intraspecific sequence distances range from 0.0 to 0.4% whereas interspecific distances range from 3.8 to 6.4% (Table 3), which indicates that species-level identification is possible with these sequences. Next, the percent distances were calculated between our data and previously reported

TABLE 3—*Percent distance matrix of four Korean Calliphorinae fly species:* Calliphora lata *(EU880183–EU880187)*, Calliphora vicina *(EU880188–EU880192),* Tricertopyga calliphoroides *(EU880176–EU880179), and* Aldrichina grahami *(EU880180–EU880182).*

	C. lata	*C. vicina*	*T. calliphoroides*	*A. grahami*	*L. illustris**
C. lata	0.0–0.1				
C. vicina	3.8–4.0	0.0–0.3			
T. calliphoroides	4.7–5.0	3.8–4.1	0.1–0.6		
A. grahami	6.0–6.2	5.8–6.0	6.2–6.5	0.0–0.1	
*L. illustris**	7.2–7.3	7.3–7.5	8.2–8.4	8.7–8.8	–

**Lucilia illustris* (L14945) is included as outgroup.

sequences. Four previously reported *C. vicina* sequences (NCBI accession numbers AY842603, AY842604, DQ345096, and AJ417702) showed only 0.2–0.6% distances from our data. Although no *C. lata* sequences are available in NCBI GenBank yet, there is a COI sequence reported as *Calliphora nigribarbis* (*C. nigribarbis* DQ345094), a synonym for *C. lata* (18) and a short sequence (304 nucleotides) of Japanese *C. lata* reported only in print by Saigusa et al. (9). DQ345094 and the Japanese *C. lata* sequence showed 0.2% and 0.7% to 1.0% distances from our *C. lata* sequences, respectively. Among three Chinese *A. grahami* sequences in NCBI GenBank (DQ345097, DQ328667, and AY818124), DQ345097 and DQ328667, both submitted by Zhu et al., were closely related to our data (0.1–0.6%). However, AY818124 diverged considerably from ours and also from two other Chinese sequences at 6.5–6.9% distances, while showing a striking similarity to *C. vicina* sequences (0.7–1.4% sequence distances). Therefore, a possibility of misidentification should be considered for AY818124. Because there are no previously reported nucleotide sequences of *T. calliphoroides*, we performed a BLAST search with our *T. calliphoroides* sequences and found that our sequences showed no more than 96% similarity with any entries in the NCBI GenBank.

The phylogenetic tree, generated from our data and previously reported sequences of Calliphorinae species, revealed a paraphyly of the genus *Calliphora* (Fig. 1). *A. grahami*, *Eucalliphora latifrons* (*E. latifrons* AF295557), *T. calliphoroides*, and *Cynomya cadaverina* (*C. cadaverina* AF259505) occupied positions scattered among the species in the genus *Calliphora*. This phylogenetic result coincides with taxonomic views on the genera *Aldrichina* and *Eucalliphora* by Rognes (19) and Whitworth (20), which consider genera *Aldrichina* and *Eucalliphora* as synonymies for the genus *Calliphora.* Our result suggests that the genus *Triceratopyga* may also be a synonymy for the genus *Calliphora.*

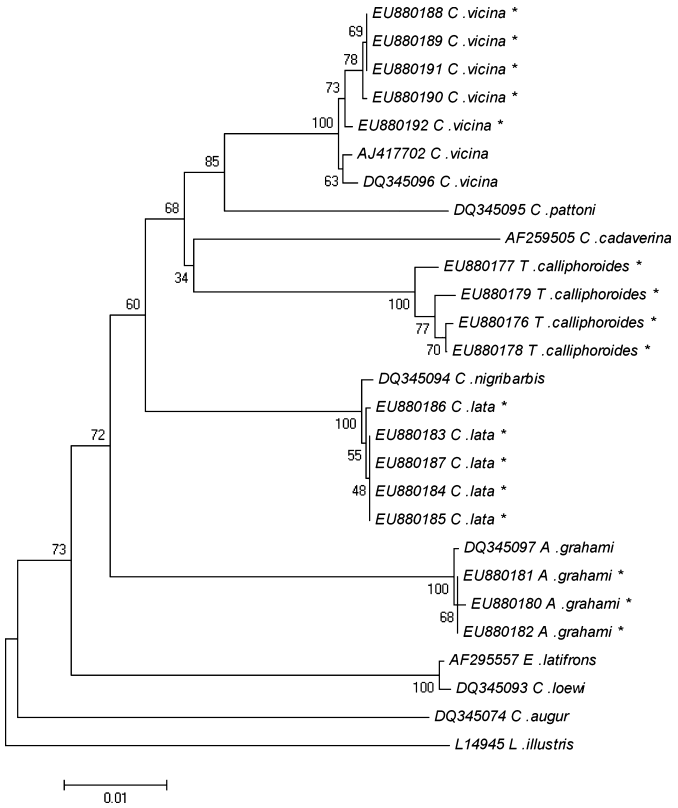

FIG. 1—*Neighbor-joining phylogenetic tree of the COI sequences of Korean Calliphorinae flies combined with previously reported data. Samples marked with "*" are from this study. Numbers on the branches are percent values from 1,000 bootstrap replicates.* Lucilia illustris *(L14945) is included as outgroup.*

In conclusion, our results demonstrate that COI nucleotide sequence analysis is suitable for species identification of Korean Calliphorinae flies. Furthermore, our result can be a reference for the future taxonomic revision in the subfamily Calliphorinae.

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