DNA barcoding of *Lucilia* blow flies (Diptera: Calliphoridae) collected in Costa Rica

Identificación molecular mediante código de barras de DNA de moscas *Lucilia* (Diptera: Calliphoridae) recolectadas en Costa Rica

Shirleny Sandoval-Arias[1], Roberto Morales-Montero[2], Emanuel Araya-Valverde[3], Erick Hernández-Carvajal[4]

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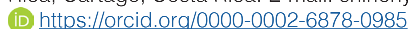
1 Máster en Ciencias Forenses. Escuela de Biología, Instituto Tecnológico de Costa Rica, Cartago, Costa Rica. E-mail: shirlenysandov20@gmail.com.

https://orcid.org/0000-0002-6878-0985
2 Perito Judicial 2. Licenciado en Biología-Zoología. Departamento de Ciencias Forenses, Organismo de Investigación Judicial (OIJ), Heredia, Costa Rica. E-mail: moralesm.roberto@gmail.com.

https://orcid.org/0000-0002-6503-2383
3 Investigador. Master en Biotecnología. Centro Nacional de Innovaciones Biotecnológicas (CENIBiot), CeNAT-CONARE, San José, Costa Rica. E-mail: earaya@cenat.ac.cr.

https://orcid.org/0000-0002-0025-5420
4 Investigador Asociado. Doctor en Biomedicina. Centro de Investigación en Biotecnología, Instituto Tecnológico de Costa Rica. E-mail: erhernandez@tec.ac.cr.

https://orcid.org/0000-0002-5585-9125

Keywords

DNA barcoding; COI; 28S; *Lucilia;* Costa Rica.

Abstract

The identification of the genus *Lucilia* is important when adults or larvae are collected in forensic cases because the post-mortem interval might be estimated. In this work, we analyzed morphologically and molecularly a panel of *Lucilia* specimens (*n* = 42) collected in Costa Rica of which 21 % of the samples correspond to flies reared from larvae taken from human corpses. Morphologically, specimens were identified as *Lucilia cuprina* [1] (33.3 % of specimens), *Lucilia eximia* [1] (33.3 %), *Lucilia purpurascens* [2] (21.4 %) or members of *Lucilia* complex (11.9 %). Molecular identification with cytochrome oxidase I (COI) or 28S ribosomal subunit regions was possible for only 43 % of the samples. Most sequences obtained with COI were according to a morphological analysis, but the 28S region lacked sufficient resolution to identify samples to the level of species except *L. cuprina*. Together, genetic and morphological data indicate that *L. cuprina* and *L. eximia* were the most commonly found species; flies reared from larvae taken from human cadavers correspond to only these species. To our knowledge, this work is the first in Costa Rica and the Centro-American region to describe cadaveric entomofauna of *Lucilia* genus, which is valuable for the development of potential forensic applications.

Palabras clave

Código de barras ADN; COI; 28S; *Lucilia,* Costa Rica.

Resumen

La identificación del género *Lucilia* es importante cuando se recolectan adultos o larvas en casos forenses porque el intervalo post-mortem puede ser estimado. En este trabajo se analizó morfológica y molecularmente un panel de especímenes de *Lucilia* (n = 42) recolectados en Costa Rica, de los cuales el 21% de las muestras corresponden a moscas criadas a partir de larvas tomadas de cadáveres humanos. Morfológicamente, los ejemplares fueron identificados como *Lucilia cuprina* [1] (33,3% de los ejemplares), *Lucilia eximia* [1] (33,3%), *Lucilia purpurascens* [2] (21,4%) o miembros del complejo *Lucilia* (11,9%). La identificación molecular, con las regiones de la citocromo oxidasa I (COI) o de la subunidad ribosomal 28S, sólo fue posible para el 43% de las muestras. La mayoría de las secuencias obtenidas con COI fueron concordantes con los análisis morfológicos, pero la región 28S carecía de suficiente resolución para identificar muestras al nivel de especies, excepto para *L. cuprina*. Juntos, los datos genéticos y morfológicos indican que *L. cuprina* y *L. eximia* fueron las especies más comúnmente encontradas; las moscas criadas a partir de larvas tomadas de cadáveres humanos correspondieron sólo a estas especies. Hasta donde sabemos, este trabajo es el primero en Costa Rica y en la región centroamericana en describir la entomofauna cadavérica del género *Lucilia*, lo cual es valioso para el desarrollo de potenciales aplicaciones forenses.

Introduction

The family Calliphoridae of blow flies is among the families most studied in the world. This family has synanthropic habitats that impact directly in forensic entomology, medical, veterinary and economic subjects [3, 4, 5 y 6]. In particular, blow flies are among the first colonizers of cadavers that might serve as a biological clock to measure the time of death and are important

in an estimation of the post-mortem interval (PMI) [3]. More than 1000 species are described for Calliphoridae; 126 species are found in the Neotropics, belonging to subfamilies Calliphorinae, Chrysomyinae, Melanomyiniae and Luciliinae [7]. Specifically, as the subfamily Luciliinae is diverse and heterogeneous, taxonomic classification becomes confusing [8]. As many taxonomic descriptions for Luciliinae blow flies lack sufficient resolution, it is difficult to verify the species that they represent without seeing the original types [6]. Inside Luciliinae, the genus *Lucilia* is forensically important; shortcomings have been described also for taxonomical identification leading to confusion and difficulty in resolving the species status for any but the most common species [6]. Accuracy is mandatory in the identification of forensically important *Lucilia* species, but can be difficult because of similarities in their morphology [9]; only experts such as taxonomists and trained technicians can identify taxa accurately through extensive experience [10]. As a DNA approach was proposed for the identification of insect species for forensic issues [11] and for species identification [12], cytochrome oxidase I (COI) has been used world-wide for molecular identification of forensically important species [9, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27]such as Calliphoridae and Sarcophagidae, provides evidence for estimation of postmortem interval (PMI, including blow-fly genus *Lucilia* [28, 29, 30, 31]. Some *Lucilia* species are so cosmopolitan that forensic entomology is specific to a locality; molecular studies are generally conducted in particular regions. The DNA of specimens sequenced from new localities might not exactly match published DNA sequences, indicating levels of variation, cryptic complex or revealing paraphyletic patterns of studied species [10, 19]. In particular, in Costa Rica, Whitworth [6] described morphologically and molecularly (using COI) specimens of *Lucilia cuprina* [1], *L. eximia* [1], *L. cluvia* [2], *L. rognesi* [6], *L. woodi* [6], *L. pulverulenta* [6] and *L. purpurascens* [2]. This extensive work, was conducted with samples collected at several locations in Costa Rica, provided not only new taxonomic keys for *Lucilia* but also vouchered sequences that we used to compare our DNA sequences. Our work seeks to analyze 42 *Lucilia* specimens in a new set collected in Costa Rica, of which 21% correspond to flies reared from larvae taken from human cadavers. The identification of these individuals provides us with valuable information about the principal species involved in the decomposition processes that are relevant for forensic purposes.

Materials and methods

Sampling and taxonomic identification of *Lucilia* species.

Adult specimens (n = 42) were obtained from a dry reference collection of the Department of Forensic Science, Costa Rica. Information on the collection sites and sampling method (where available) was annotated (data not shown). Because of the antiquity of the collection, information about the sampling method is, unfortunately, not available for all samples. For cases in which larvae were taken from human corpses, they were reared to adulthood; these adults were used in our morphological and genetic analysis. Specimens were morphologically classified by a forensic entomologist according to taxonomic keys of Vargas [32], Morales [33] and Whitworth [6].

DNA extraction and PCR amplification

Two legs of each blow fly were used for DNA isolation (DNeasy Blood & Tissue kit, QIAGEN, final elution volume 70 µL) according to the manufacturer´s instructions. Two regions were partially amplified with PCR: (i) cytochrome c oxidase subunit I (COI) and (ii) 28S ribosomal subunit from nuclear ribosomal DNA. PCR reactions for the COI region were conducted in a final volume 20 µL, containing HotStart Ready Mix (1X, Fermentas), primer LCO1490-L (5′-GTCWACWAATCATAAAGATATTGG-3′; 0.4 mM), primer HCO2198-L

(5′-TAAACTTCWGGRTGWCCAAARAATCA-3′; 0.4 mM) and the extracted DNA (2.5 μL in the range 3-15 ng/μL). PCR was performed with an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 2 min, and a final elongation step of 72 °C for 5 min. PCR reactions for the 28S region were conducted with the same volumes of COI reactions and with primers 28S-F1 (5´-GGGAGGAAAAGAAACTAACAAGG-3´) and 28S-R1 (5´-CTGTTTCGGTCTTC CATCAGGG-3´) [4]. PCR reactions were performed with an initial denaturalization step of 95 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 60 s and 72 °C for 2 min, and a final elongation step of 72 °C for 5 min. A 96-well thermal cycler (Veriti, Applied Biosytems) was used for both PCR reactions. PCR products (5 μL) were purified (Exonuclease I, 1 μL, Thermo Scientific Fermentas) and Phosphatase Alkaline (0,5 μL, Thermo Scientific Fermentas). The mixtures were placed in a 96-well thermal cycler (Veriti, Applied Biosytems) with initial temperature 37 °C for 15 min, followed by 85 °C for 15 min. Sequencing reactions were conducted with the BigDye Terminator Kit (Applied Biosystems) with final primer concentrations 0.320 μM of either forward or reverse primer. The sequencing reactions were performed in a 96-well thermal cycler (Veriti, Applied Biosytems) with the following program: 1 cycle of initial temperature 96 °C for 1 min; 15 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 75 s; 5 cycles of 96 °C for 10 s , 50 °C for 5 s and 60 °C for 90 s; 5 cycles of 96 °C for 10 s, 50 °C for 05 s and 60 °C for 2 min. Sequencing products were purified with a kit (BigDye XTerminator®, Applied Biosystems) according to the manufacturers instructions. Purified sequencing reactions were analyzed in a genetic analyzer (3130xl, Applied Biosystems).

Sequence and phylogenetic analyses

Forward and reverse strands were manually inspected (FinchTV, Geospiza); a consensus sequence was obtained in BioEdit [34]. Consensus sequences were compared on the NCBI through the function Nucleotide BLAST (Basic Local Alignment Search Tool) to identify the homology of the sequences obtained. Multiple sequence alignments of the COI and 28S regions were conducted in Mega version 6 [35] with MUSCLE option [36] using sequences from this work and also from databases GeneBank and BOLDSYSTEMS. Models of nucleotide substitution were tested in MEGA6 [35]; Tamura 3 parameters were selected to build the neighbor-joining (NJ) tree with 1000 bootstrap replicates.

Results and Discussion

Morphological identification of Lucilia species

In total, three species of forensic interest belonging to the subfamilie Luciliinae were identified morphologically: *L. cuprina* (33.3 % of total specimens), *L. eximia* (33.3 %) and *L. purpurascens* (21.4 %) (figure 1 and table 1). These species are known in Costa Rica and are distributed throughout the entire country [6, 33]. We identified also one cryptic complex assigned as *Lucilia* Complex (12.0 % of total specimens). Specimens included in this complex have phenotypical characteristics that allowed the distinction from specimens of *L. cuprina, L. eximia* and *L. purpurascens*, but we could not classify samples from the Complex all the way to the species. A new taxonomical key is reported in which new species, including *L. rognesi, L. woodi*, *L. pulverulenta* and *L. cluvia*, are described for the Neotropic region [6]. Despite these findings, some characteristics in the new taxomic key were not clearly identified in our samples; we hence kept specimens as *Lucilia* Complex.

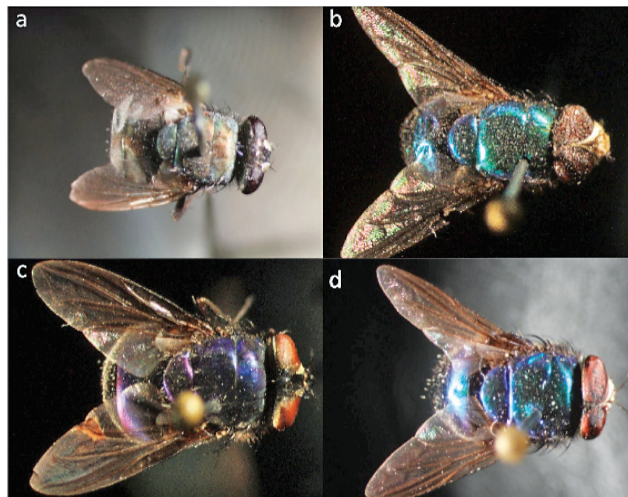


Figure 1. Taxonomic classification of specimens included in this study a) *Lucilia cuprina*, b) *Lucilia eximia*, c) *Lucilia purpurascens* and d) *Lucilia* Complex

Table 1. Taxonomic classification of 42 *Lucilia* specimens collected in Costa Rica and partial molecular identification with COI and 28S regions.

		Identification according to BLAST hits		Accession numbers in NCBI	
Taxonomic classification	Specimen code	COI	28S	COI	28S
Lucilia cuprina	1LC				
Lucilia cuprina	2LC				
Lucilia cuprina	3LC				
Lucilia cuprina	4LC				
Lucilia cuprina	5LC				
Lucilia cuprina	7LC				
Lucilia cuprina	8LC		*Lucilia cuprina*		KY798530
Lucilia cuprina	9LC				
Lucilia cuprina	10LC				
Lucilia cuprina	11LC		*Lucilia cuprina*		KY798531
Lucilia cuprina	12LC		*Lucilia cuprina*		KY798532
Lucilia cuprina	13LC	*Lucilia cuprina*	*Lucilia cuprina*	KY797311	KY798533
Lucilia cuprina	14LC				
Lucilia cuprina	29LC	*Lucilia cuprina*	*Lucilia cuprina*	KY797312	KY798534
Lucilia eximia	3LE	*L. coeruleiviridis*	*Lucilia sp.*[a]	KY797302	KY798522
Lucilia eximia	4LE				
Lucilia eximia	5LE	*Lucilia eximia*	*Lucilia sp.*[a]	KY797303	KY798523
Lucilia eximia	6LE	*Lucilia eximia*	*Lucilia sp.*[a]	KY797304	KY798524
Lucilia eximia	7LE				
Lucilia eximia	8LE		*Lucilia sp.*[a]		KY798525
Lucilia eximia	9LE	*Lucilia eximia*		KY797305	

Continue...

Continuation

		Identification according to BLAST hits		Accession numbers in NCBI	
Lucilia eximia	11LE				
Lucilia eximia	12LE				
Lucilia eximia	22LE	*Lucilia eximia*	*Lucilia sp.*[a]	KY797306	KY798526
Lucilia eximia	24LE	*Lucilia eximia*	*Lucilia sp.*[a]	KY797307	KY798527
Lucilia eximia	25LE	*Lucilia eximia*	*Lucilia sp.*[a]	KY797308	KY798528
Lucilia eximia	26LE	*Lucilia eximia*		KY797309	
Lucilia eximia	30LE	*Lucilia eximia*	*Lucilia sp.*[a]	KY797310	KY798529
Lucilia purpurascens	21LP	No significant identity [b]		KY797313	
Lucilia purpurascens	22LP				
Lucilia purpurascens	23LP				
Lucilia purpurascens	24LP				
Lucilia purpurascens	25LP				
Lucilia purpurascens	26LP				
Lucilia purpurascens	27LP				
Lucilia purpurascens	28LP		*Lucilia sp.*[a]		KY798535
Lucilia purpurascens	29LP				
Lucilia complex	31LComplex				
Lucilia complex	32LComplex				
Lucilia complex	33LComplex				
Lucilia complex	34LComplex				
Lucilia complex	35LComplex		*Lucilia* sp.[c]		KY798536

[a] Hits with large score showed several species with 100 % coverage of the input sequence and 99 % of identity with *L. mexicana, L. sericata, L. cluvia, L. illustris. L. caesar* and *L. eximia*

[b] No significant identity when BLAST was run with nucleotide collection database (nr/nt) and filtered with Organism *Lucilia* (taxid:7374). When Blast was run with the entire database nr/nt less than 87 % similarity was recorded with other organisms.

[c] Hits with large score showed several species with 100 % coverage of the input sequence and 99 % of identity with *L. thatuna, L. mexicana, L. coeruleiviridis, L. sericata, L. cluvia, L. infernalis, L. porphyrina* among others.

Alignment of COI and 28S sequences

DNA extraction was possible for all samples (42 specimens), but not all DNA were amplified with either COI or 28S. In total 12 samples were amplified for COI and 15 for 28S; all sequences were deposited in the GeneBank (table 1). This result represents rates 28.6 % and 35.7 % of PCR amplification success, respectively. Of our specimens most were collected several years ago (data not shown) and pinned for taxonomic studies; this factor degrades the DNA. Moreover, phenolic compounds were used for preservation. One limitation in the DNA barcoding surveys is the condition of a sample [10]. For this reason this method has been used mainly on only fresh specimens or samples preserved in an ideal manner for molecular work (i.e. refrigerated,

stored in ethanol or acetone and sampled in liquid nitrogen) [10, 16, 26, 30, 37]. BLAST results showed that, of nine samples morphologically classified as *L. eximia* amplified for COI, eight had significant identity to *L. eximia* with no intra-specific sequence variability. Sample 3LE was taxonomically classified as *L. eximia* but the sequence alignment had similarity to *Lucilia coeruleiviridis* (table 1). For specimens morphologically classified as *L. cuprina*, two amplified for COI with no variability in the sequences; alignment results in BLAST identified this species accordingly. Only one sequence was obtained for *L. purpurascens* that lacked significant identity with any *Lucilia* species (table 1). The 28S region lacked sufficient resolution to identify samples to the level of species except for *L. cuprina* (table 1). Sequences of *L. eximia*, *L. purpurascens* and *Lucilia* Complex showed identities to only the *Lucilia* genus level. The sequences of 28S for *L. eximia*, *L. purpurascens* and *Lucilia* Complex aligned with species such as *L. mexicana* [38] *, L. sericata* [39] and *L. cluvia*. The only sequence of *Lucilia* Complex (35L Complex) for 28S aligned for other species such as *L. thatuna* [40], *L. coeruleiviridis* [38], *L. infernalis* and *L. porphyrina* [2] (table 1). The difference in resolution when using COI and 28S was reported by Zajac *et al.* [27], who found that identification of forensically relevant Diptera species in Thailand appeared to be more conclusive with COI than with 28S. This region failed to differentiate the two closely related species *Lucilia illustris* and *Lucilia caesar* [29]. Despite 28S being inconclusive, our work is a contribution to sequence databases for future *Lucilia* DNA barcoding studies world-wide. To date only few sequences for solely 28S are available for *L. eximia* [4].

Phylogenetic analyses with COI and 28S regions

The inferred phylogenetic tree obtained with NJ (figure 2) shows the analysis for COI in which all samples of *L. cuprina* grouped with the reference sequences. The bootstrap values confirmed that molecular identification of *L. cuprina* is not complex and can be easily assayed with COI (figure 2), but identification of *L. cuprina with* conventional taxonomy was also congruent with molecular taxonomy. No monophyletic pattern using COI has been reported for *L. cuprina* [19, 25, 28, 41]. DeBry *et al*. [30] found a monophyletic group for *L. cuprina* in a vouchered collection of *Lucilia* species identified morphologically. We obtained two sequences for *L. cuprina* (13LC and 29LC) without intra-specific variation with reference sequence ASIN3389-12 (figure 2) collected in Costa Rica. The departures from monophyly can be detected only with replicate samples; additional sequences are required before the COI monophyly can be known. *L. cuprina* has shown morphological variation according to its geographic distribution [14]. The NJ tree for COI region grouped almost all our *L. eximia* samples (figure 2) with specimens collected in Costa Rica (ASIND3390-12, ASIND3391-12) and those described by Whitworth [6]; the exception was sample 3LE that grouped with accessions belonging to species newly described by Withworth [6]. This group contains also a reference accession classified as *L. eximia* from Costa Rica (ASIND3394-12). Specimens of *L. eximia* have been found to group into several discrete clusters widely separated according to geographical region; the results hence raise the possibility that *L. eximia* is a cryptic species [6]. As mentioned above, the BLAST result of sample 3LE indicated an alignment with *L. coeruleiviridis* that is impossible because this species has not been found in the Neotropical region [6]. A NJ analysis was run with reference sequences of *L. coeruleiviridis* from publications of Wells *et al*. [28], DeBry *et al*. [30] and Whitworth [6]. Sample 3LE kept grouped according to figure 2, but reference samples of *L. coeruleiviridis* also located in the same group. This result indicates a revision of conventional taxonomy considering that specimens classified as *L. eximia* might belong to a new species (*L. rognesi*, *L. woodi, L. pulverulenta*) in Costa Rica, or as stated above reflects the possibility of a cryptic species. The only sample classified as *L. purpurascens* (21LP) grouped with no reference accession (figure 2), despite our inclusion as references of several accessions of *L. purpurascens* with synonymy *L. purpurEscens*, *L. peruviana* [6].

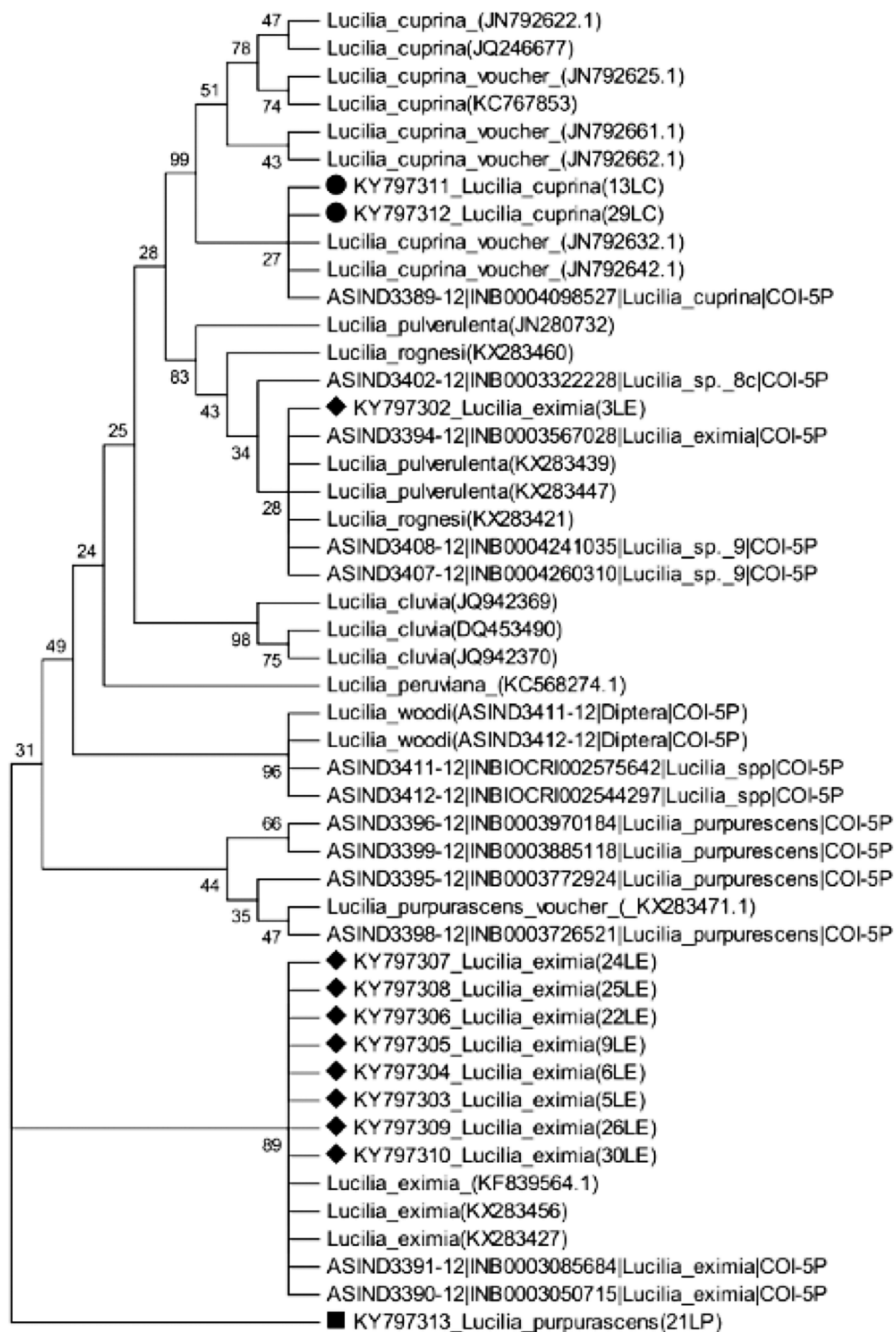


Figure 2. Phylogenetic tree of *Lucilia* specimens in this study and sequences from databases. The Neighbor-Joining method (with 1000 bootstrap replicates) was used with the COI region. The evolutionary distances were computed with the Tamura 3-parameter method and have as unit the number of base substitutions per site.

Despite samples 3LE and 21LP displaying differentially in the NJ tree, we do not declare our specimens as distinct species because more specimens of male and female blow flies are required for classification with conventional taxonomy, but the use of COI increases the accuracy for the identification of *Lucilia* species; additional knowledge other than genotype is required to shorten the list of candidate species to include only forms that can be distinguished with COI [18, 28]. The absence of concordance between morphological and molecular taxonomy in

samples 3LE and 21LP revealed the great complexity and the unresolved problem with *Lucilia* classification with conventional taxonomical keys, as occurs analogously with another *Lucilia* species [20, 30, 31]. The 28S region showed no resolution to identify some samples when sequences were aligned in GeneBank databases, except *L. cuprina* (table 1). As expected, the NJ tree (figure 3) revealed a monophyletic group with *L. cuprina*, as similarly described by Debry *et al*. [41]. A second group contained *L. eximia* (including sample 3LE) and our specimens classified as *L. purpurascens* (21LP) and *Lucilia* Complex (35LComplex).

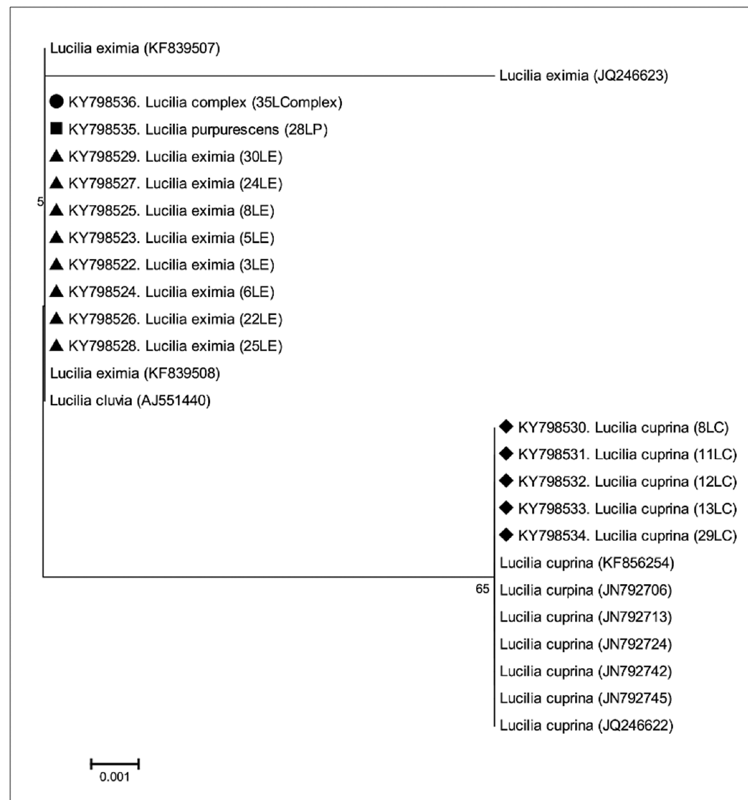


Figure 3. Phylogenetic tree of *Lucilia* specimens in this study and sequences from databases. The Neighbor-Joining method (with 1000 bootstrap replicates) was used with the 28S region. The evolutionary distances were computed using the Tamura 3-parameter method and have as unit the number of base substitutions per site.

L. eximia and *L. cuprina* are relevant species for possible forensic applications in Costa Rica

The joint analysis of the morphological and molecular data allows us to conclude that 66 % of the specimens of the OIJ collection belong to two species - *L. eximia* and *L. cuprina*. To our knowledge, the morphological and molecular analyses have not been reported for *L. eximia* and *L. cuprina* found in human cadavers, which points to these two species being two of the most relevant for forensic purposes in Costa Rica. For these two species, the necessary requirements are met for subsequent forensic application, as (i) it is possible to find larvae in human bodies, (ii) larvae can be grown to adulthood, and (iii) both morphological and molecular analyzes allow the identification of these species appropriately. Large sets of Calliphoridae samples have been studied around the world with specimens collected in varied environments and with various collection methods (i.e. nets, meat bait, liver-baited traps, pig carcases) that have increased the knowledge of the taxonomy in this family [6, 9, 13, 14, 19, 20, 30]. Larvae samples collected

from human cadavers have also been reported but in a minor proportion. In Portugal, the blowfly species *L. sericata, L. caesar* Linnaeus*, L. ampullacea* Villeneveu and *L. illustris* [39] were identified when maggots (or reared larvae) were obtained from autopsies on human cadavers [31, 42, 43]. Species *L. sericata* was found in samples from corpses in Germany [22].

Conclusion

Despite Whitworth [6] described *Lucilia* species from Costa Rica, our work enriches the knowledge of forensically important *Lucilia* species in Costa Rica. Most samples were accordingly classified with morphology and molecular analysis with COI or 28S regions, but we found contradictions for two samples (3LE and 21LP). The contradictions in morphological and molecular analyses points to the necessity of developmental characters related with the varied microclimates and geography of Costa Rica. The results of morphological and molecular analysis revealed that larvae collected from human corpses and reared to adulthood correspond to only *L. cuprina* and *L. eximia*. We identified also individuals from *L. purpurascens and Lucilia* complex. To our knowledge, this work is the first in Costa Rica and the Centro-American region to describe cadaveric entomofauna, which is valuable for the development of potential forensic applications in estimating the postmortem intervals. This work provides additional DNA sequences for *Lucilia* flies that increases the knowledge of the genetic diversity in this genus.

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