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Assessment of the *cox*1 mitochondrial DNA and *28S* ribosomal RNA genes for distinguishing newly recorded *Lucilia cuprina* Wiedemann and the established *Lucilia sericata* Meigen (Diptera: Calliphoridae) in Northwest Africa

Meriem Taleb[1] | **Halide Nihal Açıkgöz**[2]

[1]Department of Biology, Faculty of Life and Natural Sciences, University of Blida 1, Blida, Algeria

[2]Institute of Forensic Sciences, Ankara University, Mamak/Ankara, Turkey

Correspondence
Meriem Taleb, Department of Biology, Faculty of Life and Natural Sciences, University of Blida 1, BP 270, 09000 Blida, Algeria.
Email: meriemtaleb1@gmail.com

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Abstract

The mitochondrial gene cytochrome c oxidase subunit 1 (*cox1*) has long been suspected to be ineffective for species identification when employed alone. Some *Lucilia* (=*Phaenicia*) *cuprina* Wiedemann have mtDNA haplotypes closely resembling those of *Lucilia sericata* Meigen (Diptera: Calliphoridae), indicating paraphyly of *L. cuprina* with respect to *L. sericata*. Therefore, we evaluated the nuclear *28S* rRNA and the mitochondrial *cox1* genes to distinguish between *L. cuprina* and *L. sericata* using new DNA data from Northwest Africa. The current study provides the first evidence that *L. cuprina* occurs in Northwest Africa. The *cox1* and the *28S* genes were sequenced, and phylogenetic trees were constructed using the maximum likelihood method with 1000 bootstrap replicates. Sequencing yielded around 675 bp for *cox1* and 633 bp for *28S*. All the sequences were accurately identified using the BLASTn and submitted to GenBank. The *28S* analysis confirmed the two species' mutual monophyly. In contrast, the *cox1* analysis showed that *L. cuprina* is divided into two distinct clades, paraphyletic with respect to *L. sericata*. The *28S* sequences clustered together do not exhibit any geographical consistency. Despite the paraphyletic relationship between *L. sericata* and the two forms of *L. cuprina*, mtDNA appears to be useful in differentiating between these two species. However, *L. cuprina* subspecies, *L. cuprina cuprina* (Wiedemann) and *L. cuprina dorsalis* Robineau-Desvoidy cannot be differentiated using the *28S* and *cox1* genes, nor can their distinctions be assumed based on their geographic locations, especially in regions where they are found in coexistence.

KEYWORDS
first report, *Lucilia cuprina*, *Lucilia sericata*, Mediterranean blowflies, molecular identification

INTRODUCTION

The genus *Lucilia* Robineau-Desvoidy (=*Phaenicia* Meigen, Diptera: Calliphoridae) is a small group of blowflies known as the greenbottles due to their shiny green appearance (Aubertin, 1933). Among the notable species within this genus are *Lucilia cuprina* Wiedemann and *Lucilia sericata* Meigen (Diptera: Calliphoridae), which have significant medical and economic relevance (Hall & Wall, 1995). Beyond their role as carrion breeders, they are facultative parasites that cause cutaneous myiasis in sheep, among other wild and domestic animals, as well as humans (Hall & Wall, 1995; Zumpt, 1965).

Lucilia cuprina is assumed to originate from either the Afrotropical or the Oriental region (Tourle et al., 2009). This species is prevalent across most of Africa and tropical Asia, having also been introduced to Australia. Within Australia, *Lucilia cuprina* has emerged as a significant pest, inflicting myiasis on sheep (Tellam & Bowles, 1997;

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Zumpt, 1965). In addition, it occupies a central role as the primary cause of sheep myiasis in both Africa and New Zealand (Heath & Bishop, 2006), with its prevalence particularly noted in Sub-Saharan Africa (Tourle et al., 2009). It has been recorded in Europe from Malta (Hornok et al., 2023), Spain (Rognes, 1994), Portugal [unpublished data] and, on rare occasions, from the United Kingdom (Sivell, 2020), Czech Republic (Fischer & Vicha, 2003), southern Ukraine (Verves & Khrokalo, 2010). Its presence has also been documented in several Middle Eastern countries, including Turkey and Egypt (Akbarzadeh et al., 2015). *L. cuprina* is not known to exist in Italy, which is directly north of Malta (Hornok et al., 2023). The reported occurrence in Italy was attributed to a misidentification (Rognes, 2014). Owing to its preference for warmer climates, the species is unlikely to expand its range northward. However, given its initial appearances in North Africa and several southern European countries, it is probable that *Lucilia cuprina* will continue to spread across the Mediterranean Basin (Hornok et al., 2023). Data regarding the distribution of *Lucilia cuprina* in Northwest Africa are lacking. The species is universally distributed within the Afrotropical Region, with records spanning across Botswana, Cameroon, the Democratic Republic of the Congo, Ethiopia, Kenya, Lesotho, Liberia, Malawi, Mozambique, Namibia, Rwanda, Senegal, Somalia, South Africa, Tanzania, Togo, Uganda and Zimbabwe (Lutz et al., 2018).

L. sericata is a prominent pest in Europe, with its roots tracing back to the Palearctic region (Holloway, 1991). It is widely dispersed in the Holarctic region, though it has also been introduced to various regions in the southern hemisphere, including Australia, New Zealand and South Africa, where it has emerged as an important pest, comparable to its counterpart, *L. cuprina* (Zumpt, 1965).

While it is uncommon, there have been isolated reports of *Lucilia cuprina*'s association with myiasis within the Mediterranean region. Notably, there is one documented instance of human myiasis attributed to *Lucilia cuprina* in Turkey (Akinci et al., 2017), which marks its first known case in this context. Additionally, no instances of *Lucilia* spp. contributing to myiasis in humans or animals have been documented in Algeria, potentially due to the challenges associated with correctly identifying the species in that area.

Due to their predilection for consuming carrion, both *L. cuprina* and *L. sericata* larvae prove invaluable in forensic investigations, particularly for determining the Postmortem Interval (PMI). *Lucilia cuprina* has yet to be documented in Algeria, while *Lucilia sericata* has regularly been identified within forensic contexts (Taleb et al., 2018). *Lucilia sericata* is utilised in Maggot Debridement Therapy (MDT), a procedure aimed at cleansing dead tissues surrounding wounds. Conversely, *Lucilia cuprina* has been classified as unsuitable for MDT, as it persists in feeding on live tissue even after the necrotic tissue has been consumed, thereby potentially introducing microorganisms via its saliva into the wound (Sherman et al., 2000). However, subsequent evaluations have demonstrated that *Lucilia cuprina* is safe for use in MDT (Kingu et al., 2012; Paul et al., 2009; Tantawi et al., 2010).

Numerous studies, both genetic (e.g., Debry et al., 2010; McDonagh & Stevens, 2011; Tourle et al., 2009; Williams et al., 2016) and morphological (e.g., Holloway, 1991; Stevens & Wall, 1996; Williams & Villet, 2014), have been undertaken globally in an effort to discern these species that are closely related. Moreover, distinguishing between larvae of these congeners based on morphology presents a significant challenge, as they exhibit considerable similarities (Aubertin, 1933).

Because of its abundance and rapid mutation rate, mitochondrial DNA (mtDNA) is predominantly employed in forensic analyses to examine the genetics of insect specimens (Debry et al., 2010; Harvey, Dadour, & Gaudieri, 2003; Harvey, Dadour, Gaudieri, Mansell, & Villet, 2003; Junqueira et al., 2004; Li et al., 2011). PMIs may be misestimated due to misidentification when employing the mitochondrial gene cytochrome c oxidase subunit 1 (*cox1*) alone (Debry et al., 2010; Stevens et al., 2002; Tourle et al., 2009; Williams et al., 2016). As a result, Stevens et al. (2002) emphasised the need to identify blowflies using a mix of mitochondrial and nuclear markers such as the *28S* ribosomal RNA (rRNA). Furthermore, certain *L. cuprina* specimens exhibit mitochondrial DNA haplotypes that are similar to those found in *L. sericata*, suggesting that *L. cuprina* may not be a distinct lineage from *L. sericata*, but rather a paraphyletic group (Debry et al., 2010).

In this paper, we aim to evaluate the effectiveness of nuclear *28S* rRNA and mitochondrial *cox1* genes for the differentiation of *L. cuprina* from *L. sericata*. The current study provides the first evidence of *L. cuprina*'s presence in Northwest Africa, a region previously unreported in the literature on this species.

MATERIALS AND METHODS

Adult flies were captured in December 2020 using traps baited with chicken liver. The traps were left out for 1 week in a woodland habitat near the coast of Tipaza Province, North Algeria, delineated by coordinates 36°33′11.254″ N, 1°48′5.749″ E, with an elevation of 100 m above sea level. The collected flies were killed by freezing at 20°C for 1 h. *Lucilia cuprina* and *Lucilia sericata* were identified using characteristics described by Holloway (1991). Subsequently, the identified specimens were stored in a solution of 70% ethanol. Among the collected flies, three individuals of *Lucilia cuprina* were found and used for molecular identification. Several *L. sericata* specimens were identified, with three being chosen at random for DNA analysis. The samples chosen for molecular analysis were maintained at 20°C.

Each fly's hind leg was used for DNA analysis. The DNA extraction was carried out using the DNeasy® Blood and Tissue kit (Sigma-Aldrich) in accordance with the manufacturer's instructions. For the purpose of analysis, two genes were selected: *cox1* and *28S*. The *28S* gene is particularly significant as it belongs to the nuclear domain and has previously been utilised in similar research, providing a basis for comparative studies. The *cox1* gene was amplified in an area of around 750 bp using the primers LCO1490 (5′-GGTCA ACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCA GGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). A region of approximately 800 bp in domain 1–2 of the *28S* gene was amplified using the primers 5′-CCCCCTGAATTTAAGCATAT-3′ and 5′-GTTA-GACTCCTTGGTCCGTG-3′ (Stevens et al., 2002).

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TABLE 1 Specimen locality data and references for *cox1* sequences used in the phylogenetic analysis.

Species	Locality	*Cox1*
Lucilia cuprina	Algeria	PP918012
		PP918013
		PP918014
	Australia	JN792622
	Brazil	JQ246677
	Egypt	JN792625
	Hawaii	DQ453496
	Malta	OP579106
	Portugal	KY859996
	South Africa	JN792632
	Thailand	KR921650
	United States	FJ650544
Lucilia sericata	Algeria	PP916230
		PP916231
		PP916232
	Australia	JN792668
	Denmark	EF531193
	France	JN792671
	Namibia	JN792682
	South Africa	JN792690
		JN792678
	United States	FJ650561
		JN792703
	Malta	OP579110
	Portugal	JX438041
	Zimbabwe	JN792667
Lucilia cuprina × *Lucilia sericata*	South Africa	FR719164
Calliphora aldrichi (outgroup)		KX422308

TABLE 2 Specimen locality data and references for the *28S* sequences used in the phylogenetic analysis.

Species	Locality	*28S*
Lucilia cuprina	Algeria	PP922527
		PP922528
		PP922529
	Australia	KF856254
	Brazil	JQ246622
	China	MF281688
	Egypt	JN792707
	South Africa	JN792724
	Thailand	JN792741
	United States	FJ650542
Lucilia sericata	Algeria	PP922531
		PP922532
		PP922533
	Australia	JN792746
	Canada	JN792748
	China	KY197798
	France	JN792751
	Greece	JN792753
	Germany	JN792752
	Japan	JN792757
	Namibia	JN792759
	South Africa	JN792777
	UK	JQ307777
	United States	JN792779
		FJ650537
Lucilia cuprina × *Lucilia sericata*	South Africa	FR719298
		EU626536
Lucilia quadrimaculata (outgroup)		AJ558187

Amplifications by polymerase chain reaction (PCR) were performed in 25 μL volumes. The *28S* PCR mixes contained 5 μM of each primer, PCR Master Mix taq polymerase (2×) (Sigma-Aldrich), 0.5 mg/μL bovine serum albumin (BSA) (New England BioLabs), 2 μL of DNA template and sterile deionised water to a final volume of 25 μL. Thermal conditions were as follows: 94°C for 2 min, followed by 40 cycles of 15 s at 94°C, 30 s at 50°C and 45 s at 72°C, and a final extension for 7 min at 72°C. The *cox1* PCR used the same components as the *28S* PCR but with an additional 50 mM $MgCl_2$ (Thermo Fisher Scientific). The *cox1* thermocycling procedure was 95°C for 2 min, followed by 35 cycles (15 s at 95°C, 30 s at 53°C, 30 s at 72°C), and a final 7-min extension at 72°C. To confirm the success of amplification, the products of PCR were subjected to gel electrophoresis. The gel was prepared with 1.5% agarose and stained using GelRed (Biotium) to allow for the visualisation of the PCR products under UV light.

The purified PCR products were next subjected to sequencing in both forward and reverse directions, using an ABI 3130 capillary sequencer (Applied Biosystems, Foster City, CA, USA) and the same primers used in amplification. Tables 1 and 2 list the accession numbers for all new sequences deposited in GenBank. Additional DNA sequences for *L. cuprina* and *L. sericata* covering nearly 100% of the sequences from this study were obtained from GenBank and included in the phylogenetic analyses (Tables 1 and 2). The sequences were aligned and edited using BioEdit v7.0.9 (Hall, 1999). Bootstrap values were generated by resampling the datasets 1000 times. Phylogenetic analyses were performed using MEGA11 (Tamura et al., 2021) with the Maximum Likelihood method and the Tamura-Nei model.

RESULTS

Sequencing yielded around 675 bp for *cox1* and 633 bp for *28S*. All the sequences were accurately identified using the BLASTn tool from the GenBank database and submitted to GenBank (Taleb, 2024). The results of the morphological identification were validated by the molecular identification.

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All of the sequences of *L. cuprina* had a new *cox1* haplotype, with up to 99.7% (672/674 bp) sequence identity to any other sequence of this species found in GenBank.

L. sericata cox1 sequences were 100% identical to many sequences in GenBank. Likewise, the *28S* gene analyses of *L. cuprina* and *L. sericata* individuals yielded 100% identity matches to multiple sequences in GenBank, with each species' sequence group having a single haplotype.

The *cox1* phylogeny reveals that *L. cuprina* is divided into two separate clades that are paraphyletic with respect to *L. sericata* (Figure 1). *L. cuprina cox1* sequences from Algeria, Thailand, United States, Hawaii, Malta, Portugal and South Africa (*L. sericata*-like group) have clustered together in the same clade as those of *L. sericata*. Meanwhile, the hybrid sequences have grouped with the *L. sericata*-like group. *L. cuprina cox1* sequences from Brazil, Australia and Egypt have clustered separately. All *L. cuprina* sequences with mtDNA resembling that of *L. sericata* form a monophyletic and separate clade, supported by a bootstrap value of 70% (Figure 1). Our results indicate that *L. cuprina* and *L. sericata* are paraphyletic, with certain *L. cuprina* mtDNA haplotypes showing striking similarity to those of *L. sericata*. Despite this paraphyletic relationship, it appears possible to differentiate between *L. cuprina* and *L. sericata* based on mtDNA, as demonstrated by many studies (Debry et al., 2010; McDonagh & Stevens, 2011; Stevens et al., 2002; Stevens & Wall, 1995; Stevens & Wall, 1996; Wells et al., 2007; Williams et al., 2016).

The *28S* ML tree contains two well-supported clades corresponding to *L. cuprina* and *L. sericata* (Figure 2). One clade consists of *L. sericata*

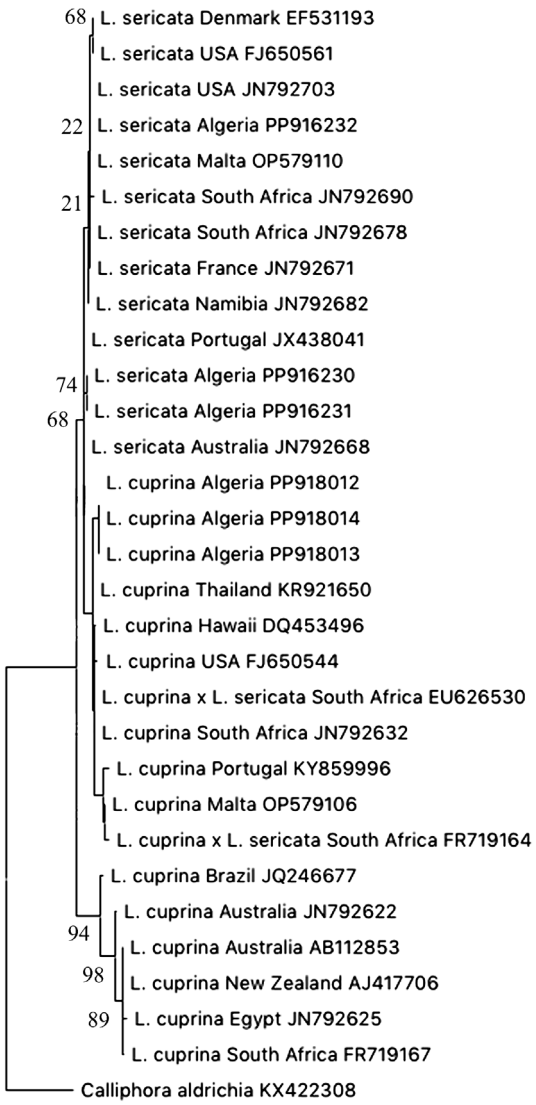


FIGURE 1 Phylogenetic tree based on the *cox1* mtDNA gene sequences of *Lucilia cuprina* and *Lucilia sericata*, generated using the Maximum Likelihood method and the Tamura–Nei model. Following the species name, each row displays the country of origin and the GenBank accession number. The analysis included 28 nucleotide sequences and 1000 bootstrap replications. The scale bar represents the number of substitutions per site.

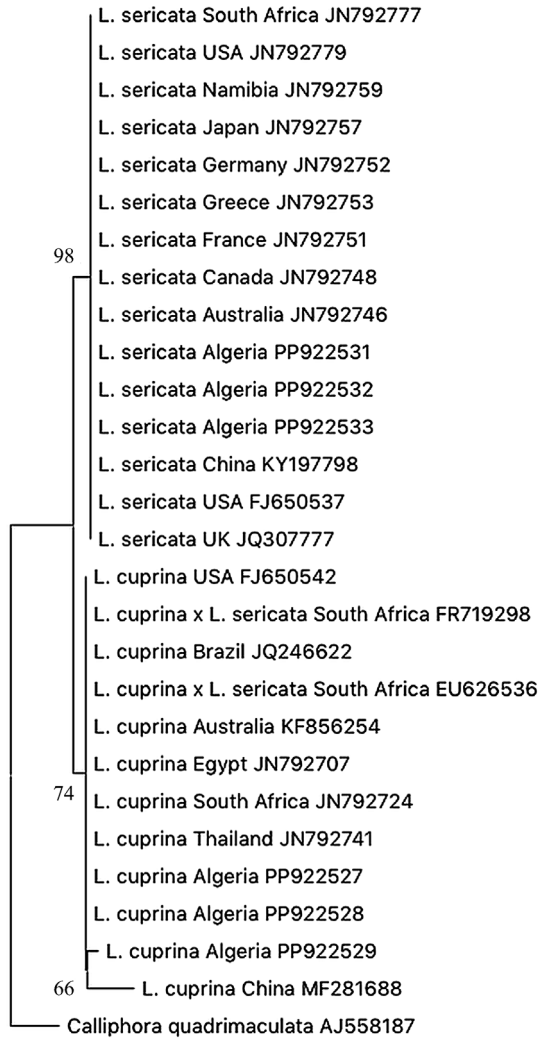


FIGURE 2 Phylogenetic tree based on the *28S* gene, generated using the Maximum Likelihood method and the Tamura–Nei model. Following the species name, each row displays the country of origin and the GenBank accession number. The analysis included 28 nucleotide sequences and 1000 bootstrap replications. The scale bar represents the number of substitutions per site.

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sequences, while the other predominantly consists of *L. cuprina* sequences, including the hybrid sequences. The *28S* sequences do not exhibit any geographic partitioning within the clades. Regardless of the geographic origin of the *L. cuprina* sample, we find reciprocal monophyly between *L. cuprina* and *L. sericata* (Figure 2), which is in agreement with previous studies (Debry et al., 2010; Stevens et al., 2002; Stevens & Wall, 1995; Stevens & Wall, 1996; Tourle et al., 2009; Wells et al., 2007; Williams et al., 2016). Compared to the *cox1* marker, *28S* data are available for significantly fewer specimens and taxa. The nuclear *28S* gene can provide data that are pertinent to the broader biological investigation of the *L. cuprina*–*L. sericata* complex; however, it is not commonly applied for forensic species identification (Debry et al., 2010).

DISCUSSION

The current study provides the first evidence of the existence of *L. cuprina* in Northwest Africa, an area where it had not previously been reported. Given its economic, agricultural, medical and forensic importance, it is crucial to monitor the range of *L. cuprina*.

The *28S* and *cox1* gene trees have consistently identified *L. sericata* and *L. cuprina* as sister species, offering robust support in numerous studies (Debry et al., 2010; McDonagh & Stevens, 2011; Stevens et al., 2002; Stevens & Wall, 1995; Stevens & Wall, 1996; Wells et al., 2007; Williams et al., 2016). Using the *cox1*, *28S* rRNA and *Period* genes, Williams et al. (2016) established *L. sericata* and *L. cuprina* as mutual sister species. These authors also noted that *L. cuprina* is paraphyletic with respect to *L. sericata* based on the mitochondrial gene *cox1* (Williams et al., 2016). Debry et al. (2010) proposed that mtDNA sequence data could distinguish between *L. cuprina* and *L. sericata*, finding that a segment of *cox1*, approximately 1200 bp in length, was sufficient for discrimination, achieving greater than 95% bootstrap support. However, our bootstrap values were lower. The nuclear gene elongation factor-1 alpha (*EF-1α*) has also been evaluated by McDonagh and Stevens (2011) but could not recover *L. sericata* and *L. cuprina* as sister species.

The biological basis for the division of the *L. cuprina* mtDNA into two clades may be more profound (Debry et al., 2010), given this species is known to be divided into two morphological subspecies. *L. cuprina dorsalis* Robineau-Desvoidy, one of the subspecies that is native to Africa, is the most common variety throughout most of Australia. This geographic distribution aligns with the mtDNA clade that is more distantly related to *L. sericata* (Debry et al., 2010). The other subspecies, *L. cuprina cuprina* (Wiedeman), is found in the Neotropics, Southern Nearctic, Southeast Asia, Hawaii and certain tropical regions of Australia (Debry et al., 2010; Norris, 1990; Waterhouse & Paramonovo, 1950).

Although hybridisation between *L. cuprina* and *L. sericata* may have given rise to the *cox1 L. sericata*-like clade, its broad geographic distribution strongly points toward a single origin rather than several instances of hybridisation (Debry et al., 2010; Nelson et al., 2012). *L. c. cuprina* has been proposed as a hybrid of *L. c. dorsalis* and *L. sericata*. *L. c. cuprina* was first recorded in Hawaii (Stevens et al., 2002; Stevens & Wall, 1996) and has since been found in Australia (Nelson et al., 2012), North America (Debry et al., 2010), South Africa (Tourle et al., 2009) and Southeast Asia (Harvey et al., 2008).

The previously established paraphyly of *Lucilia cuprina* with respect to *L. sericata* was backed by whole genome data (Nelson et al., 2012), which also indicated that *L. c. cuprina* is distinct from *L. c. dorsalis* and is more closely related to *L. sericata* than to *L. c. dorsalis* (Kapoor et al., 2023; Kapoor et al., 2024). *Lucilia c. cuprina* and *L. c. dorsalis* are two subspecies that are difficult to distinguish from one another microscopically because of their striking morphological similarities (Kapoor et al., 2024). Many of the specimens of *L. cuprina* that are listed in Genbank and the newly reported specimens here have not been classified as subspecies based on morphological characteristics. It has been suggested that the distribution of *L. c. cuprina* corresponds to the geographic origin of flies with mtDNA haplotypes very similar to those of *L. sericata* (Debry et al., 2010). Consequently, we may classify flies collected in Algeria, North America, Hawaii and Southeast Asia as belonging to the *L. c. cuprina* group, and those from other regions as part of the *L. c. dorsalis* group. However, data from South Africa (Tourle et al., 2009) challenge this straightforward concept, showing *L. sericata*-like mtDNA in *L. cuprina* flies from the same geographic range as *L. c. dorsalis*.

Debry et al. (2010) suggested that all *L. cuprina* flies within the range of *L. c. cuprina* form a monophyletic group. The South African *cox1* haplotypes were found within both *L. sericata*-like *cox1* haplotypes and the North America/Hawaii/Southeast Asia *L. cuprina* clade, which aligns with our findings. Nonetheless, the presence of *L. cuprina* with *L. sericata*-like mtDNA in South Africa does not appear to be a barrier to DNA-based species discrimination between *L. cuprina* and *L. sericata*.

Morphologically similar *L. c. dorsalis* specimens caught at the same location and time and were found by Nelson et al. (2012) to have non-monophyletic *cox1* sequences, which nested in two clades. *L. c. cuprina* has also been reported to coexist with *L. c. dorsalis* in urban areas of Australia (Kapoor et al., 2024; Nelson et al., 2012). As a result, *L. cuprina* subspecies may not be distinguished according to their geographical origin, especially from areas where they coexist. Similarly to the *cox1* results, there is no evidence of regional structure in the *28S* phylogenetic analysis that can reflect the split of *L. c. cuprina* and *L. c. dorsalis* (Debry et al., 2010; Tourle et al., 2009), which is consistent with our observations.

Nuclear findings, especially from parts of the *28S* ribosomal RNA genes, support the two species' mutual monophyly (Debry et al., 2010; McDonagh & Stevens, 2011; Stevens et al., 2002; Stevens & Wall, 1995; Stevens & Wall, 1996; Wells et al., 2007; Williams et al., 2016). In contrast, mitochondrial data, especially from the *cox1* and *cox2* genes (Stevens et al., 2002), strongly support a pattern in which *L. cuprina* is divided into two different clades that are paraphyletic in relation to *L. sericata* (Debry et al., 2010; McDonagh & Stevens, 2011; Stevens et al., 2002; Stevens & Wall, 1995; Stevens & Wall, 1996; Wells et al., 2007; Williams et al., 2016).

In analyses of both *cox1* and *28S* genes, there were no clear-cut distinctions in genetic groups formed by hybrid individuals. Several

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research efforts have documented natural crossbreeding events between *L. sericata* and *L. cuprina* (Debry et al., 2010; Stevens et al., 2002; Stevens & Wall, 1996; Tourle et al., 2009; Williams & Villet, 2013). This raises the possibility that these species are hybridising in areas where they coexist, which could have far-reaching consequences (Tourle et al., 2009). Furthermore, they have been shown in the laboratory to hybridise and produce fertile offspring, although there are no reports of this occurring naturally (Stevens et al., 2002; Stevens & Wall, 1996).

CONCLUSION

Despite the paraphyletic relationship observed between *L. sericata* and the two distinct forms of *L. cuprina*, molecular DNA analysis has proven to be a valuable method for differentiating between these species. Nonetheless, the distinction of *L. cuprina* subspecies, as determined by the analysis of the *28S* and *cox1* genes, or based on their geographical distribution, may not be reliably achieved, especially in regions where they coexist. The presence of species-specific genetic markers accurately reflects the unique genetic signatures of each species or subspecies, potentially facilitating the identification and analysis of the population structure of *Lucilia* species, particularly in areas where their habitats intersect (Kapoor et al., 2023).

AUTHOR CONTRIBUTIONS

Meriem Taleb: Investigation; writing – original draft; writing – review and editing; software; formal analysis; data curation. **Halide Nihal Açıkgöz:** Conceptualization; methodology; writing – review and editing; supervision; resources.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

DNA sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/) under the accession numbers PP918012–PP918014, PP922527–PP922529, PP922531–PP922533 and PP916230–PP916232.

ORCID

Meriem Taleb https://orcid.org/0000-0002-3017-7232

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