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A light in the dark: DNA barcoding provides new data about the taxonomy of the Italian *Luciola* (Coleoptera, Lampyridae) fireflies

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Abstract

Environmental pollution and agricultural intensification are threatening insects worldwide, and reliable taxonomy is pivotal to protect these taxa, particularly endemic species. Despite their wide distribution, lampyrid beetles (Lampyridae)—well-known as fireflies—are poorly studied in terms of taxonomy, particularly in Europe. Accordingly, as for almost all insects, the description of most species is only based on a few morphological featuresSince genetic analyses can provide valuable support in taxonomic studies, in this work, we investigated the species identity of an Italian endemic firefly, *Luciola pedemontana* (Curtis, 1843), with respect to other congeneric species, namely *Luciola italica* (Linnaeus, 1767) and *Luciola lusitanica* (Charpentier, 1825) by applying Barcoding technique. Particularly, *L. pedemontana* has been for long considered as a synonym of *L. lusitanica* or as a subspecies of *L. italica.* Italy hosts the highest diversity of firefly species in Europe, but the *Luciola* inter-specific phylogenetic relationships and species delimitations are still poorly known. With the aim to assist morphological analyses in the taxonomic characterization of species of the genus *Luciola* in Italy, we sequenced the cytochrome oxidase subunit I gene (COI) fragment of 40 individuals from 18 sites in Central Italy. Our analysis confirmed *L. pedemontana* as a well-supported monophyletic clade and as the sister taxon of *L. italica.* Furthermore, a low intraspecific genetic variation was found between *L. lusitanica* and *L. pedemontana* and between *Luciola unmunsana* + *Luciola papariensis.* Genetic data obtained for the *Luciola* species can help to improve conservation measures for *L. pedemontana,* strongly required to protect this Italian endemic taxon, which is currently threatened by light pollution and environmental alterations.

Key words: cytochrome oxidase, endemic species, insect conservation, Lampyridae, mitochondrial DNA, revised taxonomy

Introduction

Conservation biology requires sound systematics to be effective (Schaller 1977; Zachos *et al.* 2013). DNA barcoding combined with morphological features have recently helped in distinguishing among different species, by supporting

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traditional taxonomy and by enhancing the detection of cryptic taxa and hidden diversity (Tyagi *et al.* 2019; Mori *et al.* 2020; Mohd Salleh *et al.* 2023), especially within insects (Ratnasingham and Hebert 2013; Wilson *et al.* 2017). Studies on beetles have examined the effectiveness of DNA barcodes in identifying morphologically described species units (e.g., Raupach *et al.* 2010; Pentinsaari *et al.* 2014; Magoga *et al.* 2018). For example, a study by Pentinsaari *et al.* (2014) revealed that 98.3% of 1872 North European species show distinct DNA-barcode arrays and suggested the presence of at least 20 cryptic species of beetles (Coleoptera).

Besides reasonable consideration of different species (Del Cerro *et al.* 2010; Rohland *et al.* 2010; Ancillotto *et al.* 2019), taxonomic artifacts and splitting frenzy based on debatable or mild morphological features have raised the number of described animal species, often frustrating conservationists and slowing-down conservation efforts (Padial and De La Riva 2006; Mori *et al.* 2019). Despite such taxonomic inflation, boundaries at and around the species level are uncertain, also because of the high number of species concepts, making a definite, non-arbitrary species delimitation almost impossible (Zachos 2016). In other words, disentangling between splitting fairness and wrongness may be challenging and it is always case-specific (Zachos and Lovari 2013; Lorenzini and Garofalo 2015; Zachos 2018).

Fireflies (Coleoptera, Lampyridae) are a relatively small family of beetles, occurring worldwide with about 2,500 species classified in 12 subfamilies (Martin *et al.* 2017, 2019; Ferreira *et al.* 2020; Bocakova *et al.* 2022). Most fireflies emit bioluminescent signals to attract mates; thus, they can be easily detected in spring and summer nights, at least at temperate latitudes (Picchi *et al.* 2013; Branchini *et al.* 2014). Habitat loss, light pollution, and the use of pesticides represent the most important threats to Eurasian fireflies and have brought several species to a remarkable decline throughout most of their range, raising concerns about their long-term conservation (Chow *et al.* 2014; Lewis *et al.* 2020). Thus, a solid taxonomy is strongly required to address effective conservation measures, e.g., to properly conduct correct taxa identification, enabling the development of conservation strategies.

In Europe, Italy hosts the highest animal diversity (de Jong *et al.* 2014), potentially including over 20 species of fireflies (Fanti 2022, 2024), and yet little is known about their phylogenetic diversity (cf. Day *et al.* 2014). The Italian *Luciola* Laporte, 1833 (hereafter *L.*) species are *Luciola pedemontana* Motschulsky, 1853, *Luciola lusitanica* (Charpentier, 1825) and *Luciola italica* L. 1767.

Among them, the firefly *L .pedemontana* (Curtis, 1843) is currently recognized on a morphological basis as an endemic species to the Italian peninsula (Gurcel *et al.* 2020; Fanti 2022). In details, *L. italica* and *L. pedemontana* are morphologically very similar and to the moment, they can be distinguished from only an inconspicuous qualitative feature (e.g., a spot on the central part of the pronotum in *L. italica*, absent in *L. pedemontana*: Bonaduce and Sabelli 2006). Besides that morphological description, no genetic information is available for this taxon, whereas a genome assembly was produced for *L. italica* (Catalan *et al.* 2024).). Distributions of *L. italica* and *L. pedemontana* in peninsular Italy are partly overlapping in northern regions (Picchi *et al.* 2013; Fanti 2022). Namely, *L. italica* occurs in northern regions, whereas *L. pedemontana* is widespread from the Po Plain to southern regions and, possibly, to the largest islands (Camerini 2022; Fanti 2022). A reliable species splitting is pivotal for conservation issues (Zachos 2016, 2018), particularly for firefly species, which are threatened also in Italy (Picchi *et al.* 2013).

Given the widespread distribution of *L. pedemontana* in Italy and its blurred taxonomic identity, we aimed at conducting the first DNA barcoding analyses of this species, to investigate genetic species identity and its genetic distinctiveness from the similar *L. italica* and *L. lusitanica*. We analyzed COI (Cytochrome Oxidase I) gene variation in firefly samples to determine whether the barcoding diversity is strongly concordant with species units recognized in previous studies and with morphological observations.

Materials and Methods

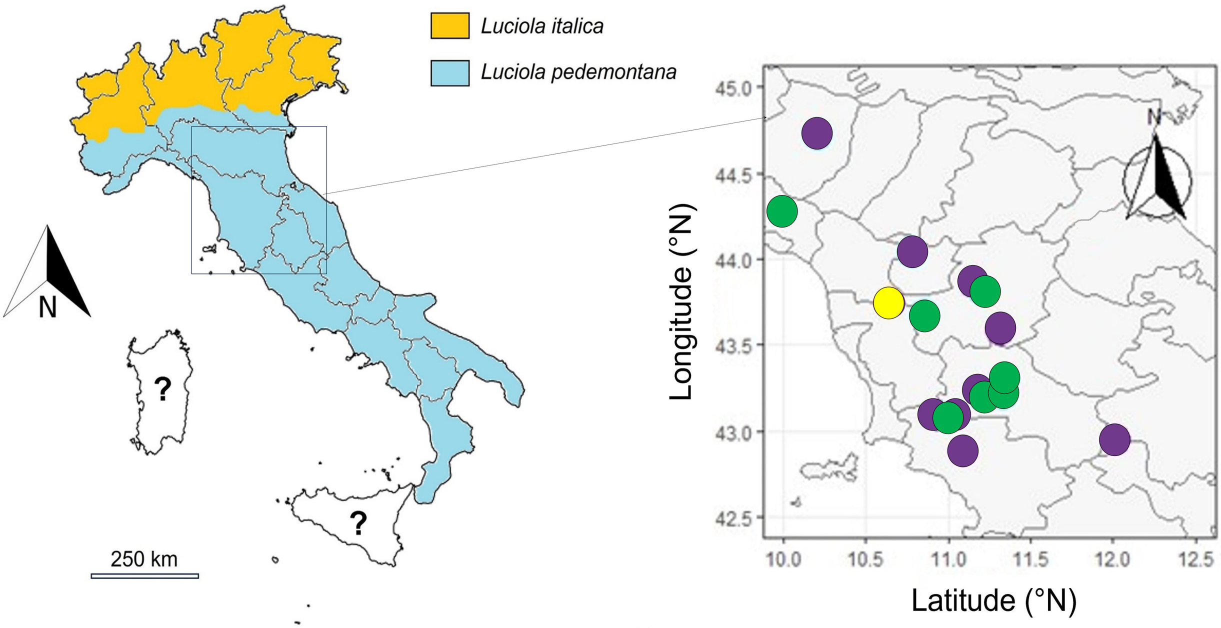
Study area

We conducted our research in Central Italy (Table S1; Fig. 2B) where *Luciola* spp. are widespread along a varied ecological and altitudinal gradient, from sea level to plain wetlands, and hills and mountains up to above sea level. (Fanti 2022).

To distinguish between *Luciola pedemontana* and *Luciola italica*, we followed morphological features described above (a spot on the central part of the pronotum in *L. italica*, absent in *L. pedemontana*: Bonaduce and Sabelli 2006) (Fig. 1)). Conversely, specimens of *L. italica* that had already been sequenced were identified by unidentified specialists from Central Europe based on their distinct central pronotum mark following Fanti (2022).

a) b)

FIGURE 1. Photos of A) *L. pedemontana* and B) *L. Italica*. A) authorized by Carlo Galletti B) authorized by Dr. Tomi Trilar Slovenian Museum of Natural History, Ljubljana, Slovenia.



a) b)

FIGURE 2. A) Known distribution of *L. italica* and *L. pedemontana* in Italy (question marks mean unknown occurrence of both species: Fanti 2022); B) sampling sites: different colors indicate individuals from different COI groups as observable in Figure 1.

Material examined

Individuals of *L. pedemontana* (identified following Porta 1929, by the lack of the spot on the central part of the pronotum) were collected in May-July 2023 (Table 1), located by walking in potential suitable environments during nighttime (about one hour after sunset), by detecting the bioluminescent signals emitted by adult fireflies. The individuals were captured by hand or with a hand-held net, with a collection limit of 1–4 samples per site to prevent overexploitation of local populations.

TABLE 1. Samples of *L. pedemontana* collected in our study (Italy, Northern Apennine region: Tuscany and neighboring regions)

Species	Location	Province	Latitude (°N)	Longitude (°E)	N samples
L. pedemontana	Prata	Grosseto	43.081087	10.984161	2
L. pedemontana	Niccioleta	Grosseto	43.087697	10.936154	3
L. pedemontana	Boccheggiano	Grosseto	43.088385	11.034160	2
L. pedemontana	Montepescali	Grosseto	42.881172	11.085253	3
L. pedemontana	Sesto Fiorentino	Firenze	43.820667	11.199669	2
L. pedemontana	Calenzano	Firenze	43.862854	11.154247	2
L. pedemontana	Greve in Chianti	Firenze	43.583843	11.311205	2
L. pedemontana	Oasi Dynamo – Limestre	Pistoia	44.038132	10.775790	1
L. pedemontana	Padule di Bientina	Pisa	43.715192	10.658801	2
L. pedemontana	San Miniato	Pisa	43.669023	10.862501	3
L. pedemontana	Ville di Corsano	Siena	43.217265	11.330004	2
L. pedemontana	Siena	Siena	43.295410	11.342660	3
L. pedemontana	Colonna Leopoldina di Montarrenti	Siena	43.233156	11.175573	3
L. pedemontana	Fornoli di Villafranca in Lunigiana	Massa Carrara	44.253823	9.968599	1
L. pedemontana	Città della Pieve	Perugia	42.952457	12.017806	3
L. pedemontana	Boschi di Carrega Regional Park	Parma	44.720033	10.208255	4

In Italy, there is no national reference law for the protection of insects and the regulation of their sampling, outside protected areas. Sampling is legally prohibited (but for authorized exemptions) only for species protected under the Habitats Directive (92/73/EU) and regional laws, which do not include *L. pedemontana*.

DNA extraction and sequencing

Sampled individuals were preserved in 96% ethanol till extraction. DNA was extracted using the Qiagen Blood and Tissue kit (©Qiagen, Inc, Tokyo, Japan). We amplified a portion (638 bp) of the mitochondrial DNA COI, by using HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') primers (Folmer *et al.* 1994). The PCR was carried out with an Eppendorf Master Cycler X50s thermal cycler in 25 µl mix including 100ng of each DNA sample, buffer 10×, 2 mM MgCl2, 200 µM dNTPs, 0.2 µM of each primer, and one unit of Taq polymerase (©Life Technologies, Waltham, Massachusetts, USA). PCR conditions included initial denaturing at 94°C for 2 min, followed by 35 cycles of 94°C for 45″, annealing at 50°C for 30″, extending at 72°C for 1 min, and a final extension to 72°C for 10 min. PCR products were run by electrophoresis on 1.5% agarose gel, containing 0.5 mg/ml of SYBR gel staining. PCR amplifications were then purified (ExoSAP-IT PCR clean-up Kit, ©Applied Biosystems, Foster City, California, USA) and sequenced through the chain termination method at the BMR Genomics (https://www.bmr-genomics.it/, Padua, Italy: accessed on 02.11.2023). Electropherograms were displayed on the Chromas 1.45 software (http://www.technelysium.com/au).

Sequences were manually corrected and analyzed with the Mega XI software (Tamura *et al.* 2021). All specimen details and sequences were uploaded to Genbank (https://www.ncbi.nlm.nih.gov/genbank. Accession numbers reported in Table S1 in Supplementary Material 1) and to the Barcode of Life Data Systems (BOLD, www.boldsystems.org: accessed on 02.11.2024) into the container project CNRIN in Italy. Firefly sequences obtained in this study were aligned with previously published COI sequences of similar species available on GenBank (http://www.ncbi.nlm.nih.gov).

Data analysis

The mean genetic inter- and intraspecific p-distances for nominal species were calculated and displayed in a pairwise distance matrix, grouping individuals firstly according to the district (Tuscany coast NW [Pisa and Massa Carrara], Firenze, Siena, Grosseto, Perugia, Parma) and then in relation with the three clades obtained by phylogenetic analyses (clade 1, 2, 3). In recent years, several methods based on molecular data have been proposed for delimiting species. To infer *Luciola* species delimitation criteria based on a partial COI gene, molecular operational taxonomic unit (MOTU) estimations were performed by employing two molecular tools: Automatic Barcode Gap Discovery

(ABGD) and Bayesian Poisson Tree Processes (PTP). The first method ABGD (Puillandre *et al.* 2012) run on the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html: accessed on 02.11.2023). The ABDG separates the species based on a range of maximum intraspecific distance. Parameters were steps = 10, X (relative gap width) = 1.5, number of bins = 20. The bPTP method was performed applying the Poisson tree process (PTP) (Zhang *et al.* 2013) based on a non-ultrametric tree run on the PTP web server (https://species.h-its.org/ptp: accessed on 02.11.2023). The maximum likelihood (ML) tree was used as the input. The PTP analysis was then performed for 100,000 generations MCMC, with a thinning value of 100 and burn-in of 0.1.

A median-joining network was also built up for *L. pedemontana* haplotypes using TCS software (Clement *et al.* 2000) based on Maximum Parsimony and graphically visualized by TCBuntu (dos Santos *et al.* 2016). JModelTEST 2.1.1 (Darriba *et al.* 2012) was used to test the most accurate model of substitution using the Bayesian Information Criterion (BIC; Schwarz 1978) and Akaike's Information Criterion (AIC), corrected for the heterogeneity between sites (gamma [G]).

In the phylogenetic reconstruction we included the available and ascertained sequences of *L. italica* and *L. lusitanica*. On the whole dataset, the TIM2+G nucleotide substitution model (transitional model: Posada 2003) was selected. We carried out a phylogenetic reconstruction by Maximum Likelihood (ML) and Bayesian Analysis (BI). We applied a Bayesian approach performed by MrBayes v 3.2.6. (http://mrbayes.sourceforge.net/download.php). Sequences of *Lampyris* spp. (Table S1 in Supplementary Material 1) were used as outgroups. We ran four Markov chains in two independent analyses for 10 million generations, sampling every 1,000 generations. The first 25% of samples were discarded as burn-in. Support node values were given as posterior probability values (pp). An ML analysis was performed using SeaView software Vers. 5 (Gouy *et al.* 2021) with the TIM2+G model and 1000 bootstrap replicates. We selected optimized choices, and we obtained the tree-searching operations by Nearest-Neighbour Interchange (NNI) and Subtree Pruning–Regrafting (SPR). Trees were visualized and edited using the FigTree vers. 1.4 software (www.tree.bio.ed.ac.uk/software/figtree: Edinburgh, UK). We followed Jusoh *et al.* (2020) for intraspecific 2% and interspecific (≥7%) distances within Luciolinae subfamily.

Results

We successfully amplified 38 sequences of *L. pedemontana,* which corresponded to 20 haplotypes. In the phylogenetic tree (Fig. 3) we found strong support for the existence of *L. pedemontana* as a well-supported clade, divergent from *L. italica* (mean genetic distance: 4.0%) and other firefly species (N = 40 sequences, N = 226 polymorphic sites, N = 97 parsimony informative sites, $\pi = 0.04$, haplotype diversity: 0.74). Conversely, this clade was weakly divergent from *L. lusitanica* (p: 2-3%). The ML and BI (shown) trees presented similar topologies, albeit different support values presented at each node (Fig. 2). In particular, the node at the basis of *L. italica* and *L. lusitanica+pedemontana* Is highly supported by BI (=0.86) but weakly by ML analysis (100). Suggesting a not reliable separation. However, the support levels at the node sustaining the *pedemontana* group are robust for both inferences (0.92/95).

The haplotypes were analysed by the species delimitation criteria: results obtained by ABGD suggested 3 MOTUs, confirming the presence of a 'barcode gap' (Fig. S1 in Supplementary Material). The three species are *L. pedemontana + L. lusitanica, L. italica* and *L. unmunsana + L. papariensis*. These two last species, described as different taxa, show a low interspecific genetic distance (2%) and have been proposed as synonyms (cf. Jusoh *et al.* 2021). The PTP method identified five MOTUs within the *Luciola* genus, with the described species *L. italica, L. lusitanica*, *L. unmunsana*+ *L. papariensis* and two taxa inside of *L. pedemontana.* (Fig. S1, Supplementary Material). The first taxonomic group inside pedemontana (1+3 in the Fig. 3) identified by PTP analyses for *L. pedemontana* is not highly supported in the phylogenetic tree (0.79/79) and comprises the subgroup 1 (Eastern) with specimens from Parma, Perugia, Siena, Grosseto, and Florence provinces and the subgroup from Padule di Bientina (Group 3, geographically and phylogenetically close to the Eastern group) one of the major wetlands in Tuscany located in the Pisa province; the Group 2 included Pisa, Massa Carrara, Siena, Grosseto and Florence provinces (Western), (Fig. 3). The same group separation was also observed in the TCS network, where le distances among the three groups exceed the 90% of connection limit and they appeared as separated networks (Fig. 4). The PTP method more sensible to small dataset and useful for taxonomic investigation is more coherent with the groups identified by phylogenetic analyses although not highly supported.

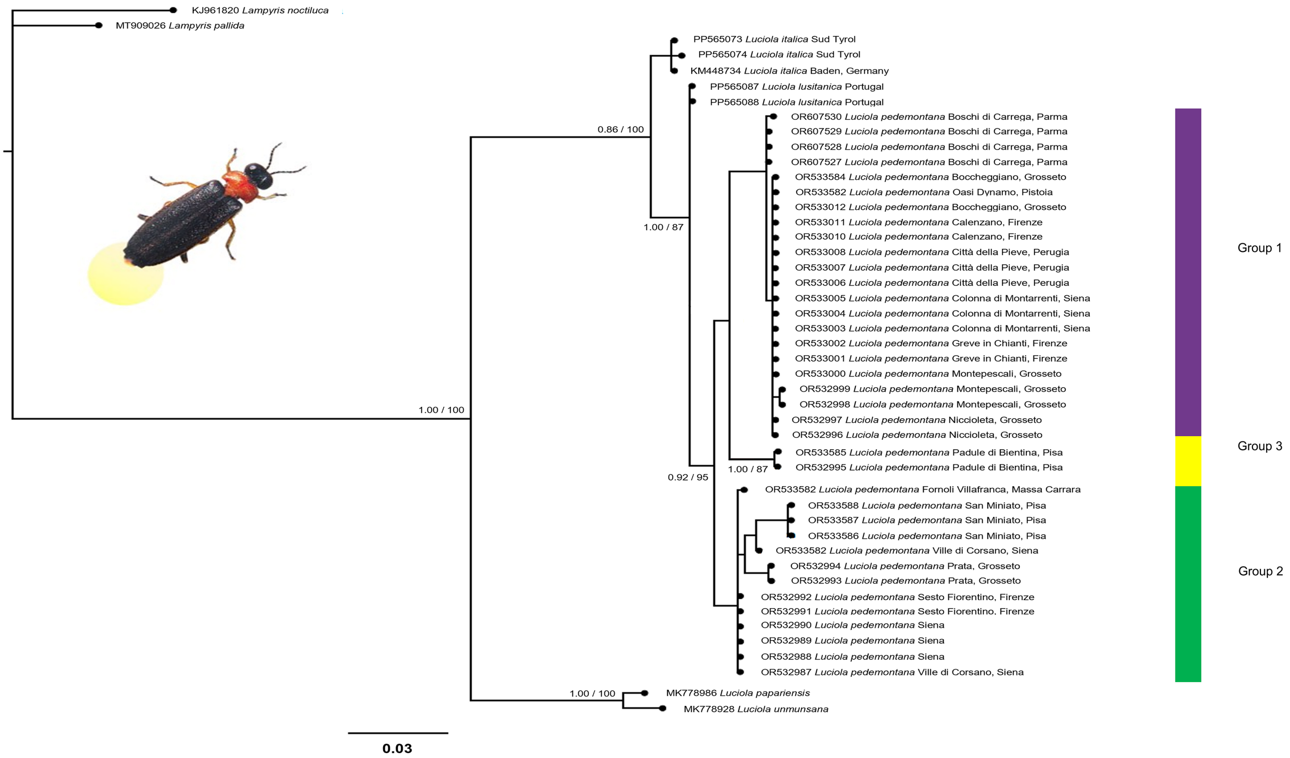


FIGURE 3. Phylogenetic BI tree inferred from COI sequences for the studied taxa. The nodal numbers are Bayesian Posterior Probabilities (BPP) and ML bootstrap values, respectively. Information for *L. pedemontana* sample used in this phylogenetic analysis (collection site, accession number) is reported in Table S1.

The genetic distances of the COI sequence between species ranged from 3.8% in average (*L. italica* and *L. pedemontana*) to 21% between the two genera *Luciola* and *Lampyris* species (Table 2). The three *L. pedemontana* groups showed a genetic distance among them of around 2.8% that appears under the threshold found for intraspecific distances in Lampyridae. Intraspecific genetic distances ranged from 0.3% in Group 1to 1.1% for Group 2 (Table 2).

TABLE 2. Uncorrected p-distances among *Luciola* spp. and closely related taxa based on COI. Groups 1,2,3 included taxa as reported in the text. In bold intraclade diversity. *LP*: *L. pedemontana.*

	LP group 1	LP group 2	LP group 3	*L. italica*	*L.lusitanica*	*L. papariensis*	*L. unmunsana*	*L. pallida*	*L.s noctiluca*
Lp group 1	**0.3%**								
Lp group 2	2.5%	**1.1%**							
Lp group 3	2.7%	2.4%	**0.9%**						
Luciola italica	4.0%	3.7%	3.9%						
L. lusitanica	2.2%	2.7%	3.1%	4.0%					
L. papariensis	13.0%	12.0%	13.0%	11.4%	13.0%				
L. unmunsana	13.0%	11.6%	12.6%	11.8%	12.9%	2.1%			
L. pallida	20.0%	19.0%	20.1%	19.6%	19.8%	18.8%	19.2%		
L. noctiluca	21.7%	21.1%	21.5%	20.3%	21.2%	20.2%	20.7%	7.7%	
L. noctiluca	21.3%	20.5%	20.9%	20.0%	20.8%	19.6%	20.0%	8.4%	0.2%

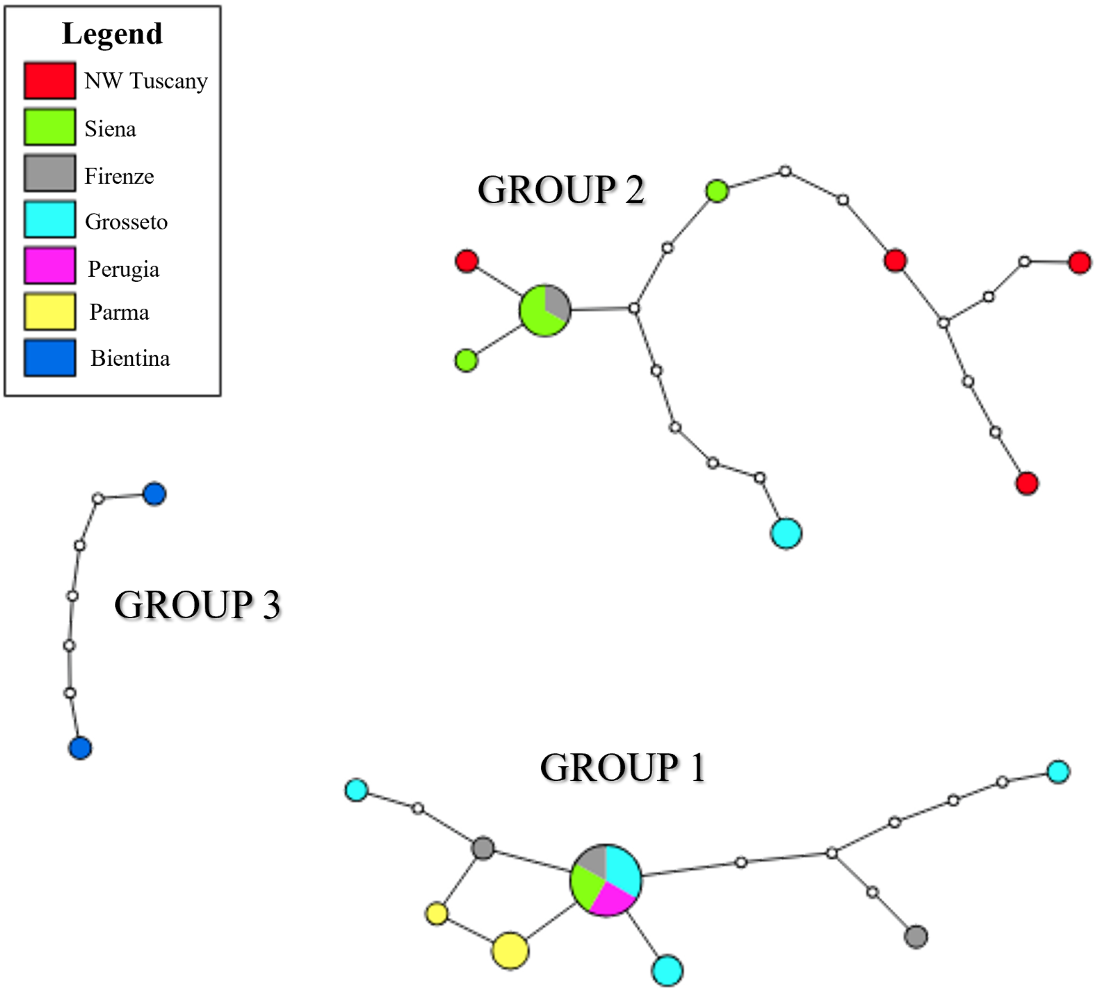


FIGURE 4. TCS parsimony network showing the relationships among *Luciola pedemontana* haplotypes. Circles represent different haplotypes. Circle size is proportional to the number of samples for each haplotype. Median vectors, which represent either extant unsampled or extinct ancestral sequences, are indicated by white circles.

Discussion

Fireflies (Lampyridae) are widely regarded as a declining and threatened species, yet their taxonomy is far from being resolved (Martin *et al.* 2019). This relatively small group of coleopterans includes over 2,500 species worldwide, but species delimitation is still poorly studied, particularly for European species (Martin *et al.* 2019). More specifically, firefly taxonomy still relies mostly on the adoption of few morphological assessments, which need to be integrated with modern DNA-based techniques (e.g., Jusoh *et al.* 2020; Roza *et al.* 2022). This is particularly evident for fireflies belonging to *Luciola* genus (Fanti 2022). Taxonomic redefinition has been carried out for several groups of species, unveiling unexpected diversity within Lampyridae, and thus triggering the description of several new species worldwide in recent years (Ferreira *et al.* 2020). So far, most genetic studies have been conducted in tropical areas (Ballantyne 2008; Ferreira *et al.* 2019, 2024; Jusoh *et al.* 2021; Silveira *et al.* 2021, Fanti 2024), where most firefly species occur, whereas very little is known on the phylogeny of temperate taxa (e.g., Japan: Bae *et al.* 2004; Maeda *et al.* 2017; North America: Stanger-Hall *et al.* 2007; Europe: Hendrich *et al.* 2015), and particularly on *Luciola* species.

In our work, we disclose the molecular evidence for monophyly of *L. pedemontana*, even though its divergence from the similar and closely related *L. italica* and from *L. lusitanica* is not high enough to sustain a distinct identity of the three taxa, based on DNA barcoding. An important environmental heterogeneity makes Central Italy one of the more environmentally diversified Italian region (Viciani *et al.* 2014), which may in turn have resulted in pronounced genetic differentiation across populations of the same species (Schmitt *et al.* 2021).

We recorded three clades of *L. pedemontana* that live in different ecotypes: the first one (Group 1, Eastern in wet areas of the Apennine ridge, the second one in dry hilly areas and mostly occurring in the Southern and Western parts of the region, and the third one in a plain wetland located in the centre of the region (cf. Ahmed 2022; Sabry *et al.* 2022). Different populations may have occupied different habitat types in evolutionary times, which may have determined this clade divergence (Schmitt *et al.* 2021). The historical distribution of forests and wetlands in the study area may have created barriers to animal movements isolating different populations, which resulted in different clade distribution, e.g. following local bottlenecks (Salomone *et al.* 2007; Milana *et al.* 2021; Schmitt *et al.* 2021; Viciani *et al.* 2024). However, although PTP analyses showed the occurrence of two main groups for *L. pedemontana,* low phylogenetic supports for at least one of the two ones (Fig. 3) suggested that all samples of *L. pedemontana* belong to the same species, as also shown by ABGD analysis. Therefore, although DNA barcoding alone may not be enough to describe a species (Zamani *et al.* 2022), we confirmed our prediction and the morphological considerations by Fanti (2022), who showed discriminant morphological differences between *L. pedemontana* and *L. italica*. Further combined analyses with morphological features and both mitochondrial and nuclear DNA markers should be conducted to definitely clarify the position of *L. pedemontana* with respect to *L. lusitanica*.

As to geographic distribution, Fanti (2022) reported that *L. pedemontana* is the commonest firefly species on the Italian Peninsula south to the Po plain, whereas *L. italica* occurs in northernmost regions (Fig. 2a). In our sample, all fireflies from the Apennines consistently belonged to the same clade, suggesting that *L. pedemontana* is the only Lampyrid from its genus occurring in central Italy. Further genetic data are needed to determine genetic distances and actual distribution of *L. pedemontana* and *L. italica* in Italy (Day *et al.* 2014). Intraspecific variability in beetles range from 1.6 to 15.8% depending on families and genera. High intraspecific genetic diversity (i.e., at least over 7-10%) may suggest cryptic species complexes (Hendrich *et al.* 2015). Several authors suggested 10% as an appropriate minimum genetic distance threshold to delimit species of flying insects using DNA barcodes, although sister species may be separated from one another also by 4-5% interspecific distances when forming well-supported clades in phylogenetic trees (Lukhtanov *et al.* 2009; Raupach *et al.* 2010; Pentinsaari *et al.* 2014; Huang *et al.* 2020; Ma *et al.* 2022). In the subfamily Luciolinae the specific threshold was established around 7%, lower than in other beetles, value higher than the levels found among the three *L. pedemontana* groups (2.8%) and between *L. pedemontana* and the other two *Luciola* species, *italica* and *lusitanica* (4% and 2-3% respectively).

Both *L. italica* and *L. pedemontana* are still commonly reported from rural and urban areas, where their conservation is primarily threatened by urban green space management (e.g., treatments against insects and terrestrial mollusks, hedgerow trimming; Lewis *et al.* 2020), light pollution (which inhibits and disrupts reproductive activity: Lewis *et al.* 2020; Vaz *et al.* 2021; Owens *et al.* 2022), and climate change (Lewis *et al.* 2020). Furthermore, in the northern Apennines, the agricultural intensification may also be responsible for declining numbers of *L. pedemontana* (Lewis *et al.* 2020). The existence of this range-restricted species seems to be confirmed by both morphological and molecular data and effective strategies for its conservation should be developed.

Fireflies are charismatic beetles with striking bioluminescent courtship displays, able to attract human attention (e.g., firefly tourism: Lewis *et al.* 2021). Thus, enhancing conservation actions to preserve their biodiversity may be well supported also by the public, and encouraged by public campaigns.

Italy seems to represent a hotspot of firefly diversity in Europe, with >20 species being reported (Fanti 2022), yet genetic sequences are only available for three (*Lampyris fuscata*, *L. italica* and *L. pedemontana*), which may limit our understanding of their actual diversity, besides impeding any conservation effort towards these beetles. A systematic and complete assessment of species identification, geographic distribution, habitat use, and threats facing Italian fireflies is thus urgently needed. As such, designing methods for rapid discrimination between similar-looking or sibling species (Zamani *et al.* 2022; Elyasigorji *et al.* 2023; Péllissié *et al.* 2023) may prove key to conducting nationwide campaigns of citizen science, which may provide valuable help in clarifying the distribution of fireflies and, consequently, foster their conservation assessment (Ancillotto and Labadessa 2023). The systematic redefinition of Italian fireflies is a pivotal element to address this conservation assessment and to determine threat categories (cf. Fallon *et al.* 2021). Future research and conservation efforts should prioritize monitoring and preserving populations of at-risk species, by identifying - and possibly mitigating - major threats, to secure their long-term conservation.

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Data availability statement Data used in this study is available in the main text. All genetic sequences are available on GenBank.

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Declarations

Conflict of interest the authors have no conflict of interest to declare.

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