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Elevation to species level of *Nebria (Oreonebria) tresignore* (Szallies & Huber, 2014) stat. nov. (Coleoptera: Carabidae), a cold-adapted, endemic and threatened ground beetle of the Italian Alps

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Abstract

Nebria (Oreonebria) tresignore (Szallies & Huber, 2014) **stat. nov.** a ground beetle living in high-altitude habitats of a restricted area of the Italian Alps (i.e., Orobic Alps), previously considered a subspecies of *N. (Oreonebria) soror* K. Daniel, 1903, was phylogenetically analyzed based on the combination of nuclear (Tpl and WG) and mitochondrial (COIa, COIb and 16S) markers. Given the morphological (male genitalia), biogeographical and ecological differences with closely related species and the newly performed phylogenetic reconstruction, its elevation to species level is proposed. This cold-adapted species is at high risk of extinction due the ongoing climate warming and glaciers retreat on the Italian Alps.

Key words: carabids, DNA analysis, glaciers, high-altitude, new species, Orobic Alps, threatened species

Introduction

Nebria Latreille 1802, is the most diverse genus within the tribe Nebriini, with almost 600 described species (subspecies included) distributed in Nearctic, Palearctic and northern Indomalayan regions (Kavanaugh *et al.* 2021). The *Nebria* genus includes predatory (i.e., opportunistic carnivores) species, across both larval and adult life stages, occasionally even consuming congeneric species (Raso *et al.* 2014). The species belonging to this genus are adapted to cool or cold environments (Ledoux & Roux 2005; Kavanaugh *et al.* 2021), and have demonstrated sensitivity to both past (Schoville *et al.* 2012) and current climate changes (Panza & Gobbi 2022). In the ongoing climatic warm stage they seem to be particularly vulnerable to variations in abiotic conditions (e.g., ground/soil temperature and humidity, gravel/

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rock rate), with changes in their body size, distribution range, microhabitat niche partitioning (Panza & Gobbi 2022; Ornaghi *et al.* 2023; Schat *et al.* 2024). High extinction risk in some montane populations, as well as local extinctions due to the habitat changes triggered by global warming, have been already documented (Kavanaugh & Schoville 2009; Pizzolotto *et al.* 2014; Panza & Gobbi 2022). The phylogeny of the supertribe Nebriitae (Coleoptera, Carabidae), based on analyses of DNA sequence data, was recently described in a study by Kavanaugh *et al.* (2021), where new species were found. Additional new species are being described, specifically from mountain areas (Sasakawa 2022).

In 2014, the subspecies *Oreonebria (Oreonebria) soror tresignore* Szallies & Huber, 2014 was described from Pizzo Tresignori in the Orobic Alps (Southern Eastern Alps, Lombardy, Northern Italy). Casale *et al.* (2021) then reassigned it to *Nebria (Oreonebria) soror tresignore* (Szallies & Huber, 2014). The authors of the subspecies compared it with the taxonomically closely related Western-Central Alpine species *Nebria (Oreonebria) angustata* Dejean & Boisduval, 1830 (Casale *et al.*, 2021) and Southern Eastern Alpine subspecies *Nebria (Oreonebria) angustata soror* K. Daniel, 1903 (Ledoux & Roux 2005), the latter elevated to species rank and placed in the genus *Oreonebria* (Szallies & Huber, 2014). On the basis of the revision performed by Kavanaugh *et al.* (2021) that downgraded *Oreonebria* to subgenus of *Nebria*, *Oreonebria (Oreonebria) soror* (K. Daniel, 1903) is *Nebria (Oreonebria) soror* K. Daniel, 1903 (Lorenz 2025).

The ecology and distribution of *Nebria soror tresignore* were subsequently investigated by Gobbi *et al.* (2018). Data collected over three years in an extended area of the Orobic Alps highlighted that this subspecies is not occurring only on the high-altitude snowfields of the Pizzo Tre Signori, as reported in Szallies & Huber (2014), but it has populations distributed among most of the Orobic Alps, never overlapping with *N. soror* s. str. distribution range (Gobbi *et al.* 2018). Indeed, the distribution range of *N. soror* s. str. is restricted to the easternmost portions of the Brescia and Garda Prealps, Southern Rhaetian Alps and Venetian Prealps (Szallies & Huber 2014) (Fig. 1).

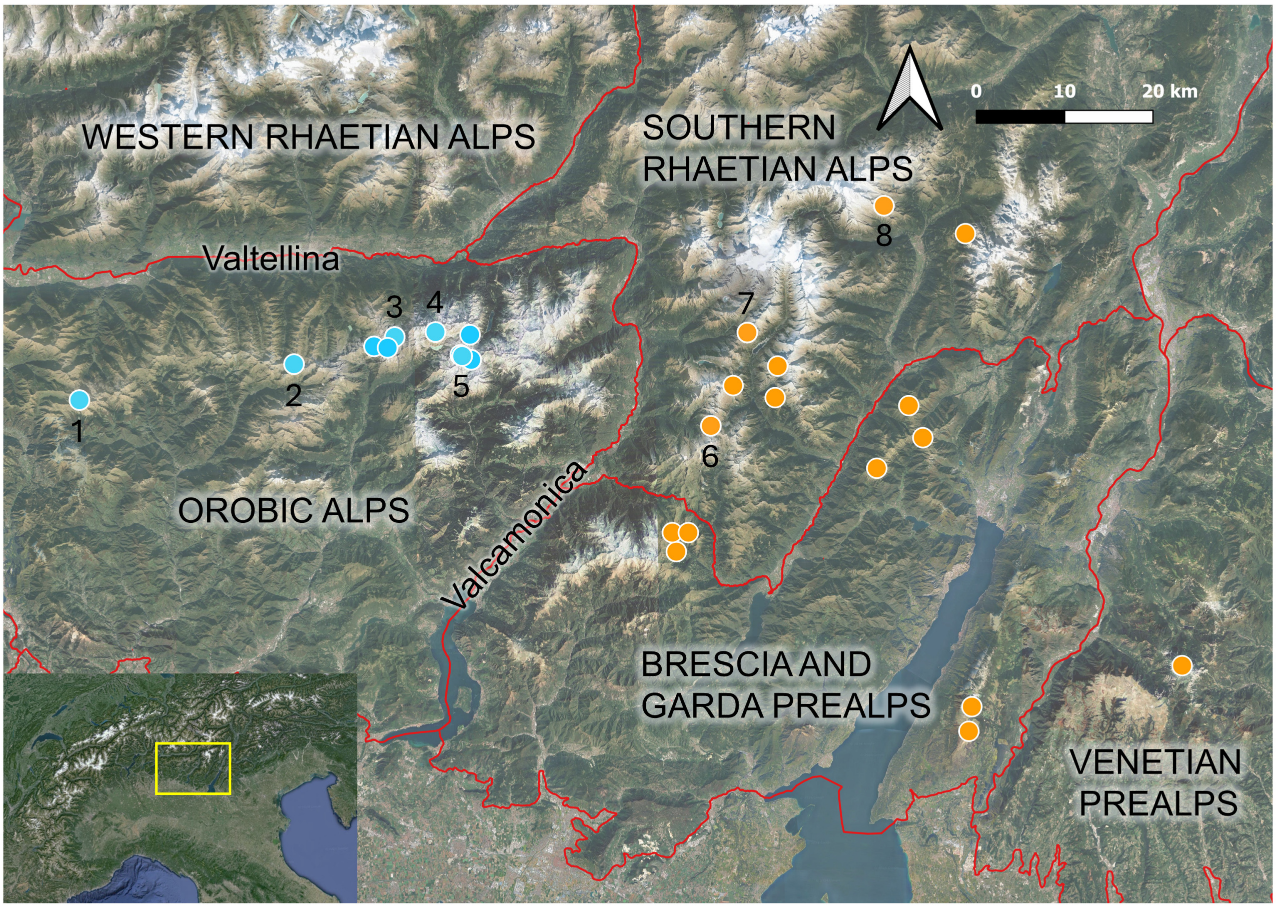

Figure 1. Distribution of *Nebria (Oreonebria) soror tresignore* (blue dots; according to Ornaghi (2019)), and *Nebria (Oreonebria) soror* s. str. (orange dots; according to Szallies & Huber, 2014 and unpublished data) within the Southern European Alps. Sampling sites of *N. soror tresignore*: 1-Pizzo Tre Signori; 2-Glacieret of Lake Publino; 3-Vedretta del Lupo; 4-Malgina Glacieret; 5-Trobio Glacier. Sampling sites of *N. soror* s. str.: 6-Listino Pass; 7-Forcel Rosso Pass; 8-Amola Rock Glacier. Red line: subsection of Alps according to SOIUSA (Marazzi 2005).

N. soror tresignore, *N. soror* s. str. and *N. angustata* are all high-altitude (above 2000 m asl), hygrophilous and cold-adapted species, often occurring on gravel-covered terrains with patchy herbaceous vegetation, scree slopes, near snowfields and on ice-related landforms (rock glaciers, debris-covered glaciers, near the tongue of debris-free glaciers) (Ledoux & Roux 2005; Tenan *et al.* 2016; Gobbi *et al.* 2018). However, *N. soror tresignore* also appears to be cryophilic (i.e., ice-related) because it has consistently been observed only within the first 50 cm from the edge of glaciers and glacierets, being absent beyond this threshold (Gobbi *et al.* 2018), and on terrains experiencing an average annual temperature of ca. 0°C or lower (Gobbi *et al.* 2014; Tampucci *et al.* 2015), while *N. soror* s. str. was sampled also on terrains with average annual temperature above 1°C (Gobbi *et al.* 2017) (Table 1).

Table 1. List of the main differences in distribution, morphology, temperature, habitat type and vegetation cover preferences between *N. soror tresignore* and *N. soror* s. str..

	N. soror tresignore **(NT)**	***N. soror*** **s.str. (NS)**	**References**
Distribution	Orobic Alps (Fig. 1)	Brescia and Garda Prealps, Southern Rhaetian Alps and Venetian Prealps (Fig 1)	NS: Szallies & Huber (2014) NT: Ornaghi 2019
Main morphological differences	Aedeagus: elongated, parallel and slightly pointed apex (Fig. 2). Elitrae: pronounced drop-shaped, with rounded narrow shoulders and very flat.	Aedeagus: short and square-built (Fig. 2) Elitrae: more evenly rounded, wider and rounded at the base with respect to NT.	NS & NT: Szallies & Huber 2014
Highest mean annual near-surface ground temperature of occurrence recorded	T = 0.5°C	T = 2°C	NS: Gobbi *et al.* 2014 NT: Gobbi *et al.* 2018
Habitat type	Glacier surface; proximity of glacier tongue; glacierets; permanent snowfields.	Rock glaciers; glacier forelands; scree slopes	NS: Tenan *et al.* 2016; Gobbi *et al.* 2017 NT: Gobbi *et al.* 2014; Gobbi *et al.* 2018; Tampucci *et al.* 2015
Ground vegetation cover (%)	Min. = 0%; Max. = 40%; Mean = 13%	Min. = 15%; Max. = 60%; Mean = 35%	NS: Tenan *et al.* 2016; Gobbi *et al.* 2017 NT: Gobbi *et al.* 2014; Gobbi *et al.* 2018, Tampucci *et al.* 2015

Given the different morphology of male genitalia (see Szallies & Huber 2014, and Fig. 2), the different distribution range of *N. soror tresignore* and *N. soror* s. str. (Gobbi *et al.* 2018; Fig. 1), the differences in their thermal, habitat and vegetation cover preferences (Table 1) and the high level of endemism that characterises the Orobic Alps (Graf *et al.* 2014), we hypothesised that these two taxa might be two different species.

Since *N. soror tresignore* was not considered in Kavanaugh *et al.* (2021), in this study we aim to test if it can be elevated to the rank of species. In addition to the information reported in Table 1, to assess its taxonomic status we use both mitochondrial and nuclear markers.

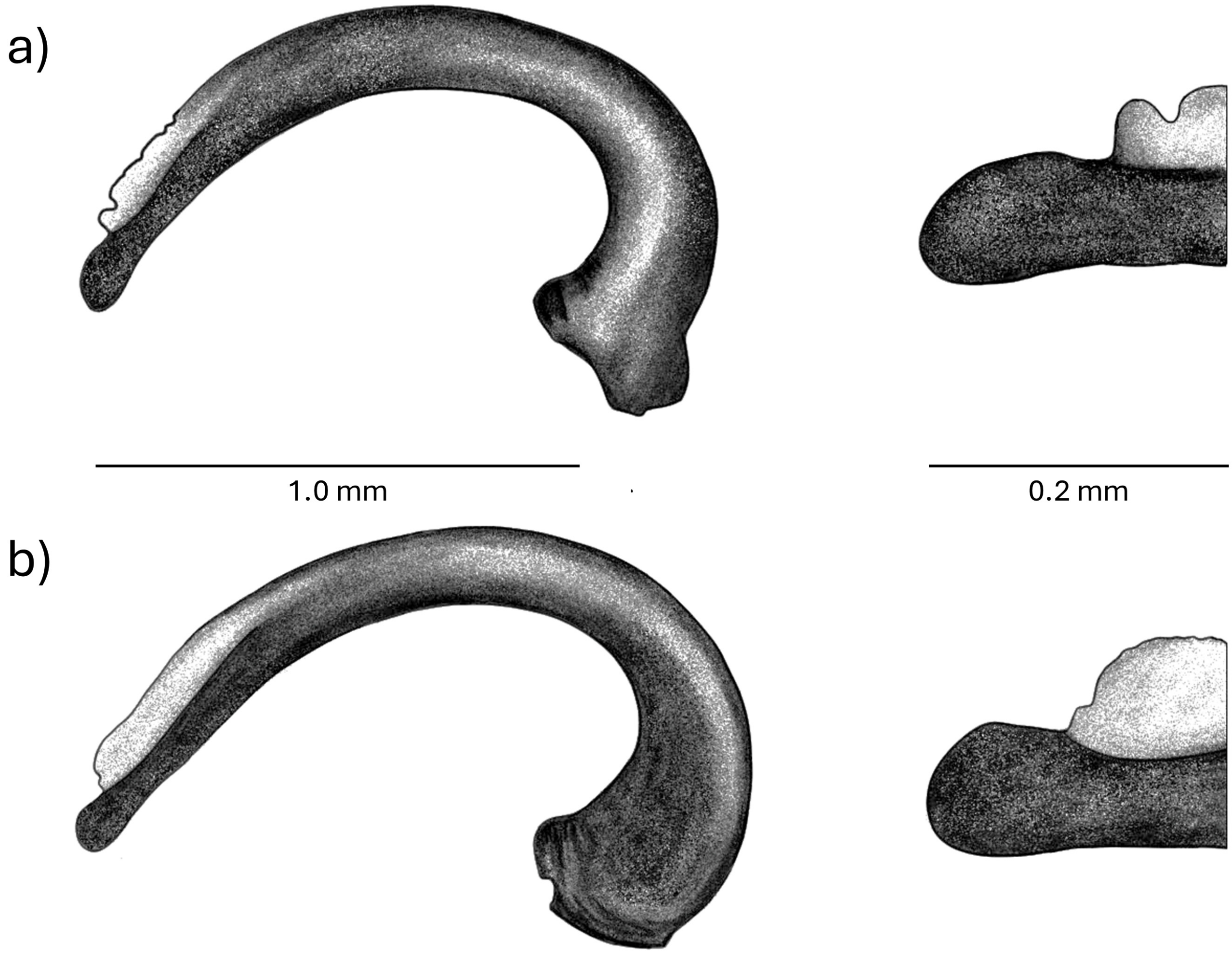

Figure 2. Shape of the aedeagus in *N. soror tresignore* (**a**) and in *N. soror* s. str. (**b**) and detail on its apex (see Table 1 and Szallies & Huber (2014) for additional info).

Material and methods

Sampling sites and specimens analysed

Five representative sites along the distribution range of *N. soror tresignore* reported by Ornaghi (2019) were selected for genetic analysis, and one specimen was analysed for each site (Table 2). A single specimen was collected from each site to minimise the impact on the populations of this subspecies (Lencioni & Gobbi 2021), which has a very restricted range and faces significant threats due to its ecological requirements (Gobbi *et al.* 2018; Ornaghi 2019). Genetic analysis of *N. soror* s. str. was conducted on 10 specimens collected within three different sites of its range (Fig. 1). Ledoux & Roux (2005) and Szallies & Huber (2014) report that some populations have individuals with two supraocular setae; this is the case of the populations sampled at Forcel Rosso and Listino Pass (Fig. 1; Table 2). For the genetic analysis of *N. soror s. str.* individuals, we therefore considered whether there was a correspondence between this morphological diversity and a potential genetic diversity. In particular, five specimens of *N. soror* s. str. have one supraocular seta and five specimens have two supraocular setae (Table 2). All 15 specimens collected from the different sites were killed by freezing and stored in 95% ethanol at -20°C.

Table 2. Samples collection data; Sample code= 'TRE': *N. soror tresignore*; 'SU': *N. soror* s. str. with one supraocular seta; 'SD': *N. soror* s. str. with two supraocular setae.

Sample	SOIUSA Subsection	Locality	Habitat	Coordinate (UTM WGS84-32T)	Altitude (m asl)	Sample code
N. s. tresignore	Orobic Alps	Trobio	glacier front	N 5101002 E 584080	2522	TRE1
N. s. tresignore		Pizzo Tre Signori	snowfield	N 5095890 E 540943	2240	TRE2
N. s. tresignore		Publino	snowfield	N 5099960 E 565245	2260	TRE3
N. s. tresignore		Malgina	glacieret	N 5103592 E 581266	2575	TRE4
N. s. tresignore		Lupo	glacier front	N 5103033 E 576650	2500	TRE5
N. soror s. str.	Southern Rhaetian Alps	Amola	rock glacier	N 5117881 E 632056	2434	SU1
N. soror s. str.		Amola	rock glacier	N 5117881 E 632056	2434	SU2
N. soror s. str.		Amola	rock glacier	N 5117881 E 632056	2434	SU3
N. soror s. str.		Amola	rock glacier	N 5117881 E 632056	2434	SU4
N. soror s. str.		Amola	rock glacier	N 5117881 E 632056	2434	SU5
N. soror s. str.		Forcel Rosso	snowfield	N 5103534 E 616586	2580	SD1
N. soror s. str.		Forcel Rosso	snowfield	N 5103534 E 616586	2580	SD2
N. soror s. str.		Forcel Rosso	snowfield	N 5103534 E 616586	2580	SD3
N. soror s. str.		Listino	snowfield	N 5092977 E 612452	2650	SD4
N. soror s. str.		Listino	snowfield	N 5092977 E 612452	2650	SD5

DNA extraction and amplification

DNA was extracted from thorax and abdomen, excluding the elytra, and the legs, following the CTAB protocol (Doyle & Doyle 1987) as adapted to insects by Bonelli *et al.* (2019). DNA was then purified using the Monarch Genomic DNA Purification Kit (New England BioLabs, Massachusetts, USA). According to the markers used for phylogenetic analysis conducted by Kavanaugh *et al.* (2021) on carabid beetles of the supertribe Nebriitae, for this study three mitochondrial (*Cytochrome A oxidase,* "COIa"; *Cytochrome B oxidase*, "COIb", and 16S) and two nuclear (*Topoisomerase*, "Tpl", and *Wingless*, "WG") genes were amplified using primers reported in Table 3 and PCR protocols reported in Table 4. Amplification products were checked on a 1.5% agarose gel and sequenced in both directions by a commercial sequencing service provider (Eurofins Genomics, Italy). Sequence data were uploaded to GenBank (accession numbers are provided in Suppl. Table 1).

Phylogenetic analysis

To clarify the phylogenetic relationships between *N. s. tresignore, N. soror* s. str. and all available related species, all the sequences within the supertribe Nebriitae from the work by Kavanaugh *et al.* (2021), defined as the *Eonebria* and *Nebria (Oreonebria)* complex, were used. Prior to phylogenetic analyses, sequence electropherograms for our newly generated *N. s. tresignore* and *N. soror* s. str. sequences were visually inspected using Benchling (2023) to detect mis-calls. Two phylogenetic reconstructions were performed, one using mitochondrial gene sequences (16S, COIa, and COIb) and one using nuclear gene sequences (Tpl and WG). For each gene sequence set, alignments were performed using Muscle included in AliView v1.28 (Larsson 2014). After manual trimming of upstream and downstream unaligned regions, multi alignments constructed from 16S, COIa, and COIb sequences were manually merged to create a concatenated mitochondrial dataset, while Tpl and WG gene sequences were merged for the nuclear dataset. In both cases, sequences representing the same haplotype in different samples were collapsed using the FaBox tool (Villesen 2007) with the function "DNA to haplotype collapser and converter". For mitochondrial genes, *N. lombarda* and *N. rettingensis* were excluded due to poor alignment. For nuclear genes, *N. sp. GAN* and *N. komarovi* were excluded because no WG sequence was available for these species. Phylogenetic analyses were performed using MEGA v11.0.13 (Tamura *et al.* 2021). The best substitution model was estimated using the appropriate MEGA function. A maximum likelihood (ML) tree was constructed for both datasets. For the mitochondrial dataset, the following parameters were used: General time reversible model, gamma distributed with invariant sites (GTR+G+I), 3 discrete gamma categories, complete deletion, 1000 bootstrap replications (all other options were left at default parameters). For the nuclear dataset, the parameters were as follows: Tamura-Nei model (T93), gamma distributed, 3 discrete gamma categories, complete deletion, 1000 bootstrap replications (all other options were left at default parameters). Genetic pairwise distance matrices were generated in MEGA v11.0.13 using the "Compute Pairwise Distances" function with the following parameters: Kimura 2-parameter model, d: Transitions + Transversions, gamma distributed, complete deletion.

Table 3. Markers used and respective primers sequences.

DNA	**Region**	**Primer Name**	**Direction**	**Sequence**	**Literature**
Mitochondrial	COIa	LCO1490 F	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* 1994
Mitochondrial	COIa	HCO2198 R	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.* 1994
Mitochondrial	COIb	Jerry F	Forward	CAACATTTATTTTGATTTTTTGG	Simon *et al.* 1994
Mitochondrial	COIb	Pat R	Reverse	TCCAATGCACTAATCTGCCATATTA	Simon *et al.* 1994
Mitochondrial	16S	16sbR F	Forward	CCGGTTTGAACTCAGATCATG	Polak *et al.* 2016
Mitochondrial	16S	16SaR aka LR-N-13398 R	Reverse	CGCCTGTTTAACAAAAACAT	Polak *et al.* 2016
Nuclear	WG	Wg550F estF	Forward	ATGCGTCAGGARTGYAARTGYCAYGGYATGTC	Wild & Maddison 2008
Nuclear	WG	WgAbRZ estR	Reverse	CACTTNACYTCRCARCACCARTG	Wild & Maddison 2008
Nuclear	WG	Wg578F intF	Forward	TGCACNGTGAARACYTGCTGGATG	Wild & Maddison 2008
Nuclear	WG	WgAbR intR	Reverse	ACYTCGCAGCACCARTGGAA	Wild & Maddison 2008
Nuclear	Tpl	TP643F	Forward	GACGATTGGAARTCNAARGARATG	Wild & Maddison 2008
Nuclear	Tpl	TP932R	Reverse	GGWCCDGCATCDATDGCCCA	Wild & Maddison 2008
Nuclear	Tpl	TP675F	Forward	GAGGACCAAGCNGAYACNGTDGGTTGTTG	Wild & Maddison 2008

Table 4. PCR conditions for each region, amplified according to the primers used. In the second column, (S) indicates a standard PCR, while (T) indicates a touchdown reaction at three different decreasing annealing temperatures. For WG and Tpl markers, a second round of cycling reactions was necessary (2' PCR). "D" indicates the initial denaturation phase; "Cycles" indicates the number of cycles; for the touchdown reactions, the number of cycles in each of the three cycling rounds is reported; "Cycles temp." indicates the temperature of each cycle phase (the second number indicates the annealing temperature used); "Cycles time" indicates the duration of each cycle; "Ext" indicates temperature and time of the final extension phase. *Wg primers = Wg550F-WgAbrz, Tpl primers = TP643F-TP932R; **WG primers = Wg578F-WgAbR, Tpl primers = TP675F-TP932R.

Region	PCR type	D. Time (min)	D. Temp. (°C)	Cycles	Cycles temp. (°C)	Cycles time (s)	Ext	Literature
COIa	(S)	4	90	35	94, 52, 72	240, 30, 45	72 °C; 10 min	Folmer *et al.* 1994
COIb	(S)	3	94	35	94, 52, 72	25, 25, 70	72 °C; 7 min	Maddison *et al.* 2012
16S	(S)	3	94	35	94, 47, 72	30, 30, 45	72 °C; 7 min	Polak *et al.* 2016
WG	1' PCR* (S)	3	94	37	94, 52, 72	25, 25, 120	72 °C; 7 min	Maddison *et al.* 2012
WG	2' PCR** (S)	3	94	35	94, 54, 72	25, 25, 150	72 °C; 7 min	Maddison *et al.* 2012
Tpl	1' PCR* (T)	3	94	6	94, 57, 72	25, 25, 120		Maddison *et al.* 2012
				6	94, 52, 72	25, 25, 120		
				36	94, 45, 72	25, 25, 120	72 °C; 7 min	
Tpl	2' PCR** (T)	3	94	6	94, 57, 72	25, 25, 120		Maddison *et al.* 2012
				6	94, 52, 72	25, 25, 120		
				36	94, 45, 72	25, 25, 120	72 °C; 7 min	

Results

Our phylogenetic analysis supports the distinction of *Nebria (Oreonebria) soror tresignore* as a separate species from *Nebria (Oreonebria) soror* s. str.. In particular, the ML phylogenetic tree constructed from mitochondrial sequences reveals two well-defined clusters corresponding to the *N. soror* s. str. group (Fig. 3a, orange background) and the *N. s. tresignore* group (Fig. 3a, blue background), with a bootstrap value of 100 at their ancestral node. Furthermore, the phylogeny also provides robust support for the separation of these two species from the ancestral *N. angustata* and its closely related species *N. austriaca* (Fig. 3a). While the ML reconstruction clearly delineates the *N. soror* s. str. and *N. s. tresignore* groups, pairwise mitochondrial genetic distances between these groups remain relatively low (0.010–0.016) compared to those observed among other recognized species included in the ML tree (Suppl. Table 2). Additionally, the subclusters within the *N. soror* s. str. group do not consistently align with the observed morphological differences (one supraocular seta vs. two supraocular setae).

Considering the nuclear dataset, the node separating the *N. soror* s. str. group (Fig. 3b, orange background) and the *N. s. tresignore* group (Fig. 3b, blue background) has a lower bootstrap value, but still suggests a separation between the two groups (bootstrap value of 78). Within the nuclear datasets, the position of the phylogenetically related species *N. austriaca* and *N. angustata* with respect to *N. s. soror* and the *N. s. tresignore* group remains unclear, with low support values (bootstrap values of 41 and 49). However, despite the lower support values in the nuclear phylogenetic tree, according to pairwise genetic distances (Suppl. Table 3), *N. s. tresignore* appears to be equally distant from *N. soror* s. str. with respect to *N. angustata* (Fig. 3b), which is considered a phylogenetically related species to both groups. Furthermore, pairwise nuclear genetic distances between *N. s. soror* and *N. s. tresignore* are comparable to, or in some cases even greater than, those observed between other pairs of taxonomically related species, such as *N. austriaca* vs. *N. diaphana* (Suppl. Table 3). This pattern aligns with the mitochondrial ML reconstruction, further supporting the distinction of *N. soror* s. str. and *N. s. tresignore* as separate species.

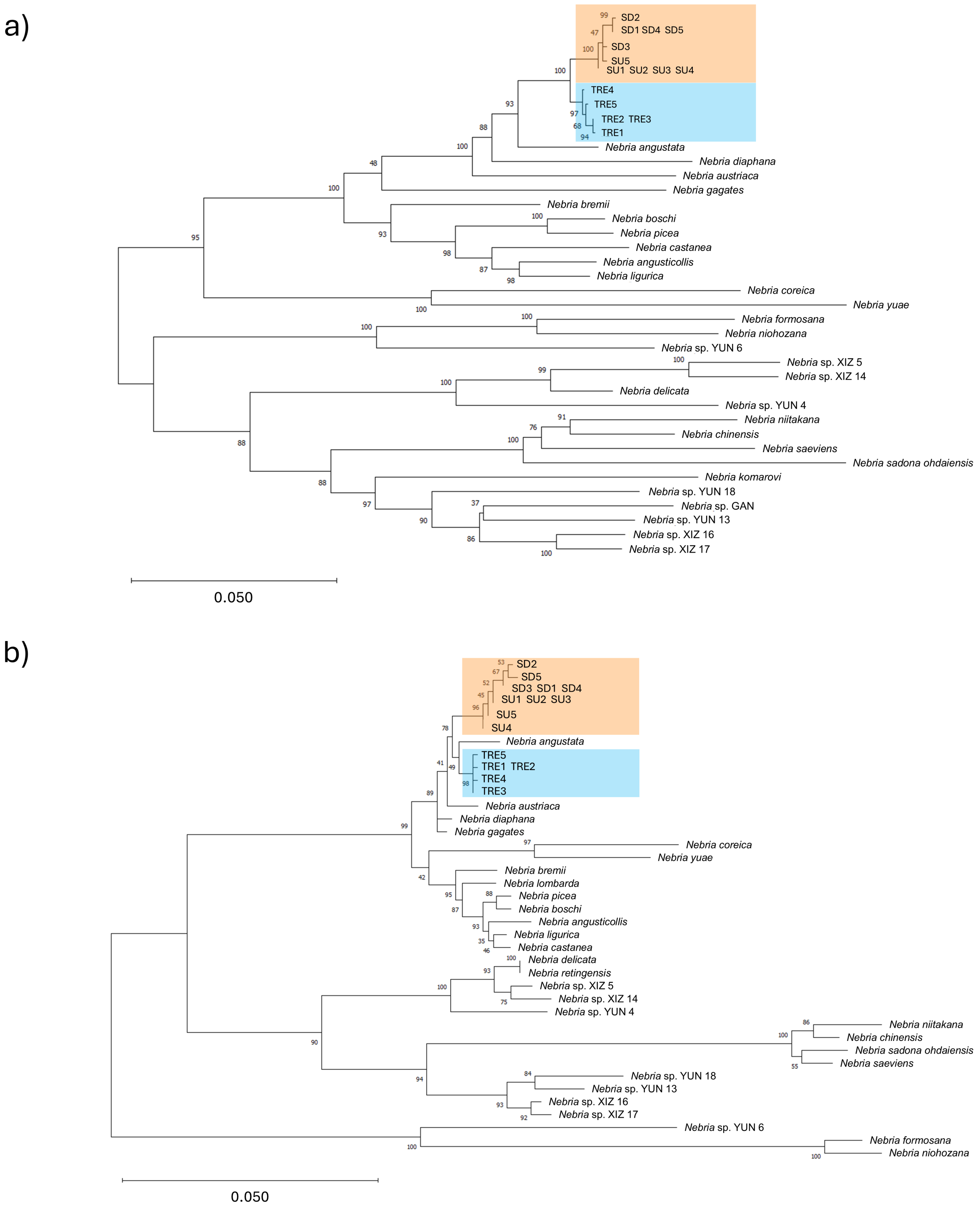

Figure 3. ML phylogenetic reconstruction of *Nebria s. tresignore* (blue background) and *N. soror* s. str. (orange background) within all publicly available species from the supertribe Nebriitae. Bootstrap values are shown on each node. (**a**) Mitochondrial genes. (**b**) Nuclear genes.

Figure 4. *Nebria (Oreonebria) tresignore* **stat. nov.** walking on the Trobio glacier. The photo, provided by Danilo Donadoni, was taken on 22th July 2018; the glacier vanished during the summer 2023.

Discussion

Our genetic analysis converged with the morphological, ecological and biogeographical evidence (Table 1) and confirmed our hypothesis: the subspecies *N. soror tresignore* can be elevated as *Nebria tresignore* (Szallies & Huber, 2014) **stat. nov.** to the rank of species.

As previously observed by Szallies & Huber (2014), both *N. soror* s. str. and *N. tresignore* are closely related taxa of *N. angustata*. The latter species presents numerous populations widely distributed in the inner part of the Western-Central Alps of Switzerland and Italy. On the basis of the geographical distribution of the considered species, we can speculate that the origin of *N. soror* s. str. and *N. tresignore* from *N. angustata* could be related to glacial and interglacial cycles that occurred during the Pleistocene period (Schmitt 2007). Indeed, during the glacier extension phases, *N. angustata* could have followed the glacier advance from the Northern Rhaetian Alps southwards, reaching Orobic Alps and Southern Rhaetian Alps. With the glaciers retreat, the Orobic and Southern Rhaetian populations of *N. angustata* could have remained isolated between each other due to the presence of Val Camonica valley and, both the populations, from the northernmost population by the presence of Valtellina valley (Fig. 1). The interrupted gene flow between these three populations possibly led to the species diversification in *N. soror* s. str. and *N. tresignore*. While it is true that the pairwise distance values observed in mitochondrial genes between the two groups are relatively low compared to those of older species included in our phylogenetic reconstruction, this may be explained by this recently hypothesized speciation event. Furthermore, lower values of mitochondrial diversity were observed between other recognized *Nebria* species that were not included in our phylogenetic analysis as not belonging to the *Eonebria* and *Nebria (Oreonebria)* complex (Kavanaugh *et al.* 2011; Kavanaugh *et al.* 2021).

According to its habitat preferences, ecology and sensitivity to climate change, *N. angustata* has been recently inserted in the Switzerland Red List of Carabid beetles and classified as NT (Near Threatened) (Chittaro *et al.* 2024). Considering the ecology of the studied species, the fragmented distribution of the sub-populations and the ongoing glaciers retreat in the areas of occurrence, the extinction risk for *N. soror* s. str. and *N. tresignore* is to be considered very high (Gobbi *et al.* 2018), and likely they are—on the European Alps—Near Threatened, too. Specifically, *N. tresignore* seems particularly vulnerable in relation to climate warming as glaciers and glacierets on Orobic Alps are few with a limited surface and are likely to disappear within a few decades because of the overall low elevation of the Orobic Alps (Smiraglia & Diolaiuti 2015) (Fig. 4).

Given the peculiar ecology of *N. tresignore*, populations of this species are supposed to migrate uphill, but due to its inability to fly (it is a wingless species) and its cryophilic needs (Table 1), *N. tresignore* may not be able to migrate long distances to find other suitable habitats or to locate suitable refugia—such as rock glaciers—where species may retreat or migrate to (Scotti *et al.* 2013; Keppel *et al.* 2015; Brighenti *et al.* 2021).

As future work, the existing morphological characters within the *N. soror* s. str. sub-populations warrant further investigation, as our genetic analysis was unable to clearly distinguish individuals with one versus two supraocular setae. Additionally, implementing a molecular clock approach will be crucial for accurately estimating divergence times, thereby providing deeper insights into the evolutionary history of these species.

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