

New insights on *Troglophilus* (Orthoptera Rhabdiphoridae) species distribution in the westernmost area of their main range (Northern Italy)

Marco BONELLI¹, Silvia MESSINETTI², Felicita SPREAFICO³

¹Department of Biosciences, University of Milan, Milan, Italy

²Department of Environmental Science and Policy, University of Milan, Milan, Italy

³Department of Earth and Environmental Sciences, University of Milano-Bicocca, Milan, Italy

Abstract

The genus *Troglophilus* Krauss includes species with a rather limited dispersal ability and a wide distribution range, making it a good model for biogeographic, phylogeographic and phylogenetic investigations, with growing interest in recent years. However, a crucial prerequisite for addressing biogeographic issues is the knowledge about species complete distribution range. In particular, *Troglophilus cavicola* (Kollar) and *Troglophilus neglectus* Krauss are the species of the genus *Troglophilus* with the widest distribution in Europe, where their main areal extends westward to Northern Italy, but their precise distribution and range limits are still uncertain. In this study, the presence of these two species, which overwinter in hypogean habitats, was investigated in caves of the westernmost area of Northern Italy (Lombardy, Provinces of Lecco and Como) where populations belonging to the genus *Troglophilus* were reported. The individuals of these populations were described by previous literature as *T. cavicola* or as *Troglophilus* sp. without species determination. In this work, an in situ morphological identification of all individuals detected in the surveyed caves, and a molecular identification on 12 specimens through the COI barcoding, were performed. An effective, fast, simple, economical and easily reproducible protocol for DNA extraction was optimized for this taxon. The presence of *T. cavicola* in the investigated area was confirmed and the presence of *T. neglectus* was reported for the first time. These new data allowed us to hypothesize different scenarios about their dispersal route in Lombardy. Moreover, these new findings must be considered to define sampling plans to elucidate the biogeography of this genus.

Key words: barcoding, biogeography, biospeleology, cave crickets, species range limits, *Troglophilus cavicola*, *Troglophilus neglectus*.

Introduction

An essential goal of biogeography is to “discover why organisms are distributed the way they are today” (Platnick and Nelson, 1978; Ebach *et al.*, 2003; McDowall, 2004) and a crucial prerequisite for addressing this question is to accurately determine the range borders of species (Zeisset and Beebee, 2001). Moreover, the knowledge of distribution boundaries is fundamental for establishing a proper sampling plan for biogeographic, phylogeographic, and phylogenetic analyses because a lack of samples from areas where the species is present could produce incomplete or non-representative results. The importance of judicious sampling to address phylogeographic and phylogenetic issues using molecular data has been stressed in the literature, and it has been demonstrated that phylogeographic studies that do not sample sufficiently across the distribution range of a species are prone to obtain results that are at best incomplete, and potentially spurious (Avendaño *et al.*, 2017). Furthermore, delimiting the distribution range of species has fundamental implications for our understanding of biodiversity and for decision-making in conservation (Mota-Vargas and Rojas-Soto, 2012).

The genus *Troglophilus* Krauss belongs to the family Rhabdiphoridae Walker, a monophyletic group of Orthoptera with a discontinuous distribution across temperate regions of both hemispheres (Jost and Shaw, 2006; Karaman *et al.*, 2011). These organisms can be

found in areas rich in shelters that can protect them during overwintering and during the dry summer season: such shelters are very abundant in karstic regions, but they can also be found in non-karstic areas (Karaman *et al.*, 2011). According to present knowledge, the genus *Troglophilus* is distributed only in Europe and Western Asia. This genus comprises 21 valid species (Allegrucci *et al.*, 2017), 10 of which have been described in the last 15 years. As stated by Allegrucci *et al.* (2017), the genus *Troglophilus* could be a suitable model for phylogeographic investigation; indeed, there has been a growing interest in this genus in recent years, addressing biogeographic, phylogeographic, and phylogenetic issues (Karaman *et al.*, 2011; Kaya *et al.*, 2013; Taylan *et al.*, 2013; Allegrucci *et al.*, 2017). Nonetheless, works to precisely determine the range borders of *Troglophilus* species are missing and Karaman *et al.* (2011) even consider the data in the literature concerning the distribution of *Troglophilus* species to be unreliable.

As far as is known, *Troglophilus cavicola* (Kollar) and *Troglophilus neglectus* Krauss are the two most widely distributed species in Europe, from the West Balkans to Italy (Karaman *et al.*, 2011). Their recent distribution out of the Balkans, in the west, is considered to be shaped by their postglacial expansion (Karaman *et al.*, 2011). Both species have a two-year life cycle, reaching the sexual maturity during the second year (Lipovšek *et al.*, 2016). They overwinter twice in hypogean habitats, while during the favourable season they are active in

epigean habitats (Pehani *et al.*, 1997; Lipovšek *et al.*, 2011; 2016; Di Russo *et al.*, 2014). Artificial and natural caves are the only subterranean refuges where they can be easily detected during overwintering (Karaman *et al.*, 2011).

The western borders of their main distribution areas are thought to be in Northern Italy (Karaman *et al.*, 2011), not considering reports from places separated from their main ranges by distance and by the Alps, such as Mayen in Germany (Kiefer *et al.*, 2000), where they allegedly were deliberately introduced (Pfeifer *et al.*, 2011 as cited in Baur and Güttinger, 2013), and Wartau in Switzerland (Baur and Güttinger, 2013). In Italy, the westernmost region in which both species were detected is Lombardy. Regarding the distribution of *T. cavicola* in this region, many interesting articles have been published based on data collected more than 30 years ago (e.g., Capra, 1951; 1959; Bini and Ferrari, 1971; Banti and Bini, 1978; Banti *et al.*, 1981; 1991; Comotti, 1983; 1986) but only a few scattered, more recent reports exist. According to the literature, the western distribution border is in the Provinces of Lecco and Como, with the westernmost populations located in the Triangolo Lariano, the peninsular area between the two branches of Lake Como (also known as Lario). Regarding *T. neglectus*, there is a lack of information about its possible distribution in Lombardy. This region was historically considered out of the range of this species, whose distribution was believed to be more eastern and limited to the Trentino Alto-Adige, Veneto, and Friuli-Venezia Giulia regions (Baccetti and Capra, 1969; Baccetti, 1982; Fontana *et al.*, 2002; Rampini and Di Russo, 2012). Nevertheless, the species was found also in Lombardy (Di Russo *et al.*, 2008), in a single cave, the “Buco del Corno” cave (LoBg 1004, Municipality of Entratico, Province of Bergamo) in 1995 and 1997 (G. Comotti, personal communication, 2017). The report from the “Buco del Corno” cave was the westernmost finding of *T. neglectus* in Northern Italy, about 70 km west of the *T. neglectus* populations located between the east side of Lake Garda and the Adige Valley in Trentino (Baccetti and Capra, 1969), and about 50 km east of the westernmost findings of *T. cavicola*, in Triangolo Lariano. Lastly, there are reports from caves in Lombardy where the specimens observed were just described as belonging to the genus *Troglophilus* without any determination of the species (Bini and Ferrari, 1971; Gagliardi, 1989).

To contribute to define the distribution ranges and limits of *T. cavicola* and *T. neglectus*, the presence of these species was investigated in the westernmost area of Northern Italy (Provinces of Lecco and Como), where populations of this genus were reported. Caves both with and without past records of *Troglophilus* presence were surveyed. The previous literature reports about the presence of *Troglophilus* in these provinces were based only on morphological data. In addition to classical morphological determination based on morphological characters, molecular analyses were performed to confirm the species identification of the specimens found during this work.

Materials and methods

This study was conducted in the prealpine area of Lecco and Como Provinces (Lombardy, Northern Italy). The topography of these provinces is characterized by the presence of freshwater bodies and mountain reliefs sloping down to hills and plains in the southern part, with flat areas strongly urbanized. The local temperate climate is influenced by the particular setting of these elements. Lake Como, Lake Garlate, and River Adda create a “water continuum” that separates the Bergamo Prealps to the east and the Como Prealps to the west (Marazzi, 2002). The investigated area extends from the Western Bergamo Prealps to the Triangolo Lariano (figure 1). From a geological point of view, the study area belongs to the southern prealpine belt, where superficial and deep karstic phenomena are widespread. Indeed, in the provinces of Lecco and Como more than 2,000 caves have been inventoried so far, according to the regional database “Catasto Speleologico Lombardo”. Their distribution reflects the geological arrangement of the calcareous lithologies of the Mesozoic sedimentary succession of Lombardy, in particular the ones that are more prone to dissolution. Moreover, during Quaternary intense glaciations influenced the geomorphology of the territory and in some cases the development of karstic phenomena (Bini *et al.*, 1998; 2009).

The first step of the present work was to collect data about past records of *Troglophilus* specimens in the Provinces of Lecco and Como (figure 1, table 1).

All these records are from caves located from the Western Bergamo Prealps to the Triangolo Lariano. The entrances to these caves are located in submontane or montane altitudinal zones and most of them (83.3%) have a horizontal development.

In the present study, a total of 33 caves located in the same altitudinal zones of previous records were surveyed (table 2), including more than 50% of those in which the presence of *Troglophilus* was already reported. Since most of the past reports were from caves with a horizontal development, and considering logistical limitations to perform a complete survey in vertical caves, only horizontal caves were chosen. Surveys were conducted between November 2016 and April 2017, during the overwintering period of the two species under investigation (Di Russo *et al.*, 2008; Lipovšek and Novak, 2015), when individuals can be detected in caves.

During the favourable season, *Troglophilus* individuals are active in epigean habitats and can move away from the cave entrance (Pehani *et al.*, 1997; Lipovšek *et al.*, 2011; 2016; Di Russo *et al.*, 2014). This opens up the possibility for interbreeding between individuals from different caves that, in this way, can belong to the same population. However, little is known about the epigean maximum distance from caves reached by cave-inhabiting Rhaphidophoridae (Taylor *et al.*, 2007) and no information is available for the genus *Troglophilus*. In the case of *Ceuthophilus secretus* Scudder, another cave-inhabiting species belonging to the same family, individuals can move for foraging up to 120 m from the cave entrance (Taylor *et al.*, 2004; 2007). According to

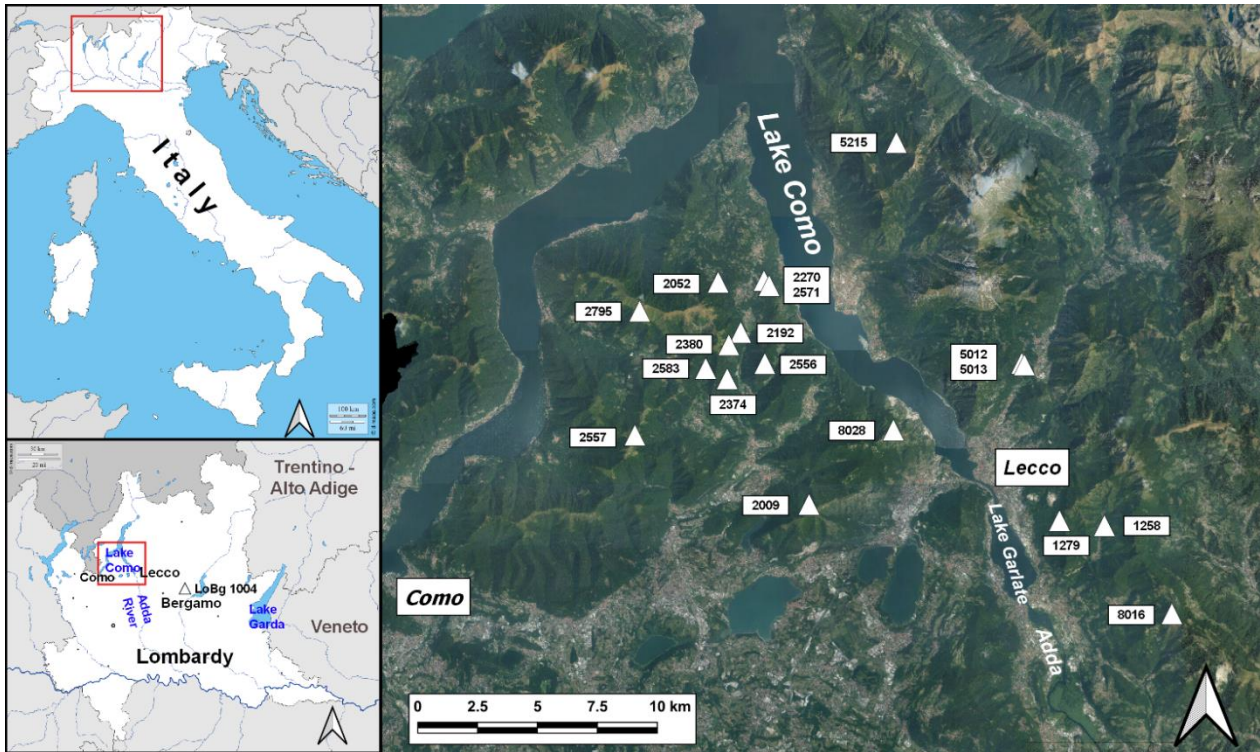


Figure 1. The investigated area. White triangles indicate the caves with past records of *Troglophilus* in the study area. The reported cave codes are the inventory codes of Catasto Speleologico Lombardo. Lombardy and Italy maps: www.d-maps.com; investigated area map: Map Data Google 2018.

these observations, possible meeting and interbreeding could occur between individuals coming from different caves up to 240 m distant from each other. In the present study, therefore, *Troglophilus* individuals collected from close caves (< 240 m) were considered as belonging to the same population, while caves more than 240 m apart were treated as hosting individuals belonging to separate populations. Caves were thus divided into groups accordingly. Each cave was visited twice by two observers at a time, recording the presence or absence of *Troglophilus*. Surveys were conducted during the day.

All individuals found, located in positions where they could be reached by the observers, were sexed and identified alive at species level on site, with the help of macrophotography techniques. Diagnostic characters provided by Karaman *et al.* (2011) were used to identify adult specimens, while the dichotomous key provided by Baccetti and Capra (1969) was employed for the nymphs. In particular, the structure and the shape of the tenth tergite, considered among the only reliable morpho-anatomical characters by which species can be distinguished (Karaman *et al.*, 2011), was considered. Body size and, for females, level of development of the ovipositor were used to distinguish between adults and nymphs.

Moreover, barcoding techniques were used to confirm the species identified on a morphological basis. For this purpose, in each group of caves, each detected *Troglophilus* species was sampled, collecting a total of 6 nymphs and 6 adults for each species.

The collected specimens were killed by freezing. Then they were dissected, and the body was conserved in 75%

ethanol, while legs were preserved in anhydrous ethanol and stored at $-20\text{ }^{\circ}\text{C}$ to perform further molecular identification. All samples are deposited at the Department of Biosciences, University of Milan, where the laboratory work was carried out. Genomic DNA was extracted from the proximal portion of the last pair of legs following a CTAB protocol (Doyle and Doyle, 1987). The tissue was dried in a thermoblock at $37\text{ }^{\circ}\text{C}$ to allow the ethanol to evaporate. After adding $50\text{ }\mu\text{L}$ of extraction buffer (1.4 M NaCl; 20 mM EDTA; 100 mM Tris pH 8.0; 2% CTAB) and $5\text{ }\mu\text{L}$ of 20 mg/ml proteinase K for protein degradation, the samples were left in a water bath at $56\text{ }^{\circ}\text{C}$ overnight. One volume of chloroform was added to each sample and then all were centrifuged at $11,500 \times g$ for 5 minutes at a temperature of $4\text{ }^{\circ}\text{C}$. The spin separated two phases: a supernatant with the DNA and a lower phase that contained proteins and polysaccharides. After collecting the supernatant, DNA was precipitated by two successive washes: in absolute ethanol and 70% ethanol, and then centrifuged for 15 and 5 minutes at $4\text{ }^{\circ}\text{C}$ and $11,500 \times g$, respectively. After the ethanol had evaporated, the pellet containing the DNA was diluted in $25\text{ }\mu\text{L}$ of molecular biology grade water. The amount of DNA extracted was checked via electrophoresis on 1% agarose gel. A 470-bp sequence of the mitochondrial COI region was obtained using the primer set C1-J-1859 (5'-GGAACnGGATGAACAGTAT-3') and C1-N-2329 (5'-ACTGTAAATATATGATGAGCTCA-3') modified from Simon *et al.* (1994) with a final concentration of $0.25\text{ }\mu\text{M}$ each. The following PCR conditions were used: an initial DNA denaturation at $94\text{ }^{\circ}\text{C}$ for 3 minutes followed by 35 cycles at $94\text{ }^{\circ}\text{C}$ for 30 seconds, $53\text{ }^{\circ}\text{C}$ for

Table 1. Past records of *Troglophilus* in the investigated area. *During this work it was assessed that LoCo 2271 and LoCo 2571, as well as LoCo 2270 and LoCo 2572, are synonyms for the same cave.

Cave code	Cave name	Coordinates (UTM WGS84- 32T) Altitude (m a.s.l.)	Cave development	Record	References
LoCo 2009	El Füs (Caverna Fusa)	N 5076349 E 523157 955	vertical	<i>Troglophilus</i> sp.	Bini and Ferrari, 1971
LoCo 2052	Büs di Pegur (Buco delle Pecore)	N 5085620 E 519364 950	horizontal	<i>T. cavicola</i>	Banti <i>et al.</i> , 1981
LoCo 2192	Grotta del Fo' di Barni (Büs dela Pissalonga)	N 5083528 E 520304 767	horizontal	<i>T. cavicola</i>	Cappa, 1962; Bini and Ferrari, 1971
LoCo 2374	Cunicolo Bur Burino	N 5081588 E 519743 778	horizontal	<i>T. cavicola</i>	Banti <i>et al.</i> , 1981
LoCo 2380	Grotta dei Quattro Passi	N 5083006 E 519810 950	horizontal	<i>T. cavicola</i>	Banti <i>et al.</i> , 1991
LoCo 2556	Büs del Negrin	N 5082226 E 521313 1060	horizontal	<i>Troglophilus</i> sp.	Gagliardi, 1989
LoCo 2557	Voragine di Monte Bul (Abisso di Monte Bül)	N 5079238 E 515891 1375	vertical	<i>T. cavicola</i>	Speleo Club CAI Erba and Speleo Club "I Protei", 1983; Banti <i>et al.</i> , 1991
LoCo 2271 (LoCo 2571)	Spaccatura a SE del Buco del Palo* Grotta Madonna delle Selve)	N 5085705 E 521336 635	horizontal	<i>T. cavicola</i>	Banti <i>et al.</i> , 1981
LoCo 2583	Grotta degli Aspidi	N 5082022 E 518847 920	horizontal	<i>Troglophilus</i> sp.	Gagliardi, 1989
LoCo 2795	Pozzo nella Valle di Lot (Abisso l'Altro Mondo)	N 5084414 E 516090 1500	vertical	<i>T. cavicola</i>	Aimar <i>et al.</i> , 2011
LoLc 1258	Büsa a la Calchera dii Sing	N 5075462 E 535486 650	horizontal	<i>T. cavicola</i>	Parenti, 1976; Banti and Bini, 1978
LoLc 1279	Büsun di Trécc	N 5075670 E 533610 770	horizontal	<i>T. cavicola</i>	Comotti, 1986
LoLc 2270 (LoCo 2572)	Buco del Palo* Grotta dell'Asee)	N 5085680 E 521277 665	horizontal	<i>T. cavicola</i>	Capra, 1959; Bini and Ferrari, 1971; Banti <i>et al.</i> , 1981
LoLc 5012	Grotta di Val Naone	N 5082236 E 532035 810	horizontal	<i>T. cavicola</i>	Comotti, 1986
LoLc 5013	Grotta del Formaggee	N 5082167 E 532145 768	horizontal	<i>T. cavicola</i>	Comotti, 1986
LoLc 5215	Grotta dietro l'Armo	N 5091451 E 526802 1505	horizontal	<i>T. cavicola</i>	Catasto Speleologico Lombardo, 2016
LoLc 8016	Grotta del Tesoro	N 5071784 E 538301 1370	horizontal	<i>T. cavicola</i>	Catasto Speleologico Lombardo, 2016
LoLc 8028	Grotta dei Tassi del Moregallo	N 5079454 E 526678 1122	horizontal	<i>T. cavicola</i>	Catasto Speleologico Lombardo, 2016

Table 2. Caves surveyed for the presence or absence of *Troglophilus* in the present study with the relative inventory numbers of Catasto Speleologico Lombardo.

Group	Cave code	Cave name	Coordinates (UTM WGS84-32T)	Altitude (m a.s.l.)
a	-	Spring Catchment	N 5083589 E 520433	680
	LoCo 2192	Grotta del Fo' di Barni	N 5083528 E 520304	767
b	LoLc 2765	Primo Buchetto di Cricri	N 5080884 E 539326	1036
	LoLc 2766	Secondo Buchetto di Cricri	N 5080879 E 539332	1040
	LoLc 2655	Grotta nel Vallone	N 5080877 E 539259	975
	LoLc 8040	Mollaci	N 5080834 E 539292	990
c	LoLc 5012	Grotta di Val Naone	N 5082237 E 532036	810
	LoLc 5013	Grotta del Formaggee	N 5082167 E 532145	768
	LoLc 5014	Grotta del Partigiano	N 5082001 E 532019	852
d	LoLc 2270	Buco del Palo	N 5085680 E 521277	665
	LoLc 2271	Spaccatura a SE del Buco del Palo	N 5085705 E 521336	635
	LoLc 2722	Grotta Riparo Irene	N 5085625 E 521324	678
	LoLc 8063	Free Camping Miralago	N 5085702 E 521304	640
e	LoLc 3728	Buco di Grao	N 5075521 E 534222	840
	LoLc 8019	Caverna di Grao	N 5075374 E 534116	900
f	LoLc 8059	Antro del Tatzelwurm	N 5076126 E 533294	665
	LoLc 8060	Phreatichthys Aprilis	N 5076120 E 533329	705
	LoLc 8061	Edera Rock	N 5076151 E 533317	699
	LoLc 8067	Vanth	N 5076067 E 533404	730
	LoLc 8068	Charun	N 5076056 E 533408	732
	LoLc 8069	Frattura delle Ossa	N 5076007 E 533428	750
not grouped	LoLc 1258	Büsa a la Calchera dii Sing	N 5075462 E 535486	650
	LoLc 1279	Büsun di Trécc	N 5075670 E 533610	770
	LoLc 1308	Büs de Carigun	N 5080822 E 537810	993
	LoCo 2052	Büs di Pegur	N 5085621 E 519364	950
	LoLc 2243	Riparo della Treminoeula	N 5077684 E 526629	575
	LoLc 2263	Grotticella del Versante Sud del Monte Rai	N 5076388 E 524398	1025
	LoLc 2273	Buco della Sabbia	N 5074980 E 525313	450
	LoCo 2556	Büs del Negrin	N 5082226 E 521313	1060
	LoLc 2611	Risorgente sopra Casa Alpe	N 5075294 E 530221	400
	LoLc 2668	Büs di Cavrecolo	N 5082016 E 538406	1094
	LoLc 8028	Grotta dei Tassi del Moregallo	N 5079454 E 526678	1122
	LoLc 8041	Fessura dei Ritornanti	N 5084878 E 535183	1150

30 seconds, and 72 °C for 45 seconds, and then by a final extension of 10 min at 72 °C. Amplification products were checked on a 1.5% agarose gel. A commercial sequence service provider (Eurofins Genomics, Italy) performed the sequencing, employing the same primers used for the amplification. Trace files and sequence data were uploaded to GenBank (Accession numbers are provided in table 3). To confirm the morphological identification, each barcode sequence was queried using the BOLD Identification Engine with the Species Level option (Ratnasingham and Hebert, 2007). A p-distance metric with pairwise deletion was used to compare sequences (Collins and Cruickshank, 2013). To further confirm the identification, a neighbour-joining tree was reconstructed, integrating our sequences with the ones already available on Genbank [*T. cavicola* accession numbers: KY412387, KY412388, KY412389, KY412390,

Table 3. List of GenBank accession numbers of barcoded specimens.

Species identification	Sequence id	GenBank accession number
<i>T. neglectus</i>	5013n2	MH785260
<i>T. neglectus</i>	2192n1a	MH785261
<i>T. neglectus</i>	2192n1b	MH785262
<i>T. neglectus</i>	5014n2a	MH785263
<i>T. neglectus</i>	2556n1	MH785264
<i>T. neglectus</i>	5014n2b	MH785265
<i>T. cavicola</i>	2668c1	MH785266
<i>T. cavicola</i>	2192c1a	MH785267
<i>T. cavicola</i>	2192c1b	MH785268
<i>T. cavicola</i>	8040c2	MH785269
<i>T. cavicola</i>	2052c2	MH785270
<i>T. cavicola</i>	8028c2	MH785271

KY412391, KY412392 from Allegrucci *et al.* (2017) and *T. neglectus* accession number: EU938374 from Fenn *et al.* (2008)]. *Dolichopoda geniculata* (Costa) (Orthoptera Rhaphidophoridae) (GenBank accession number: AY793616.1), *Dolichopoda ligustica* Baccetti et Capra (Orthoptera Rhaphidophoridae) (GenBank accession number: AY793605), and *Gryllus bimaculatus* De Geer (Orthoptera Gryllidae) (GenBank accession number: AY793605) were used as outgroups. Confidence in estimated relationships of NJ tree topologies was evaluated by a bootstrap analysis with 1,000 replicates (Felsenstein, 1985).

Results

All the data collected concerning the presence of *Troglophilus* in the examined caves are summarized in figure 2 and table 4. *Troglophilus* individuals were found in 36.4% of the investigated caves. Both *T. cavicola* (figure 3a, 3c) and *T. neglectus* (figure 3b, 3d) were detected and identified during the surveys, thanks to the morphological characters described by Karaman *et al.* (2011) and Baccetti and Capra (1969). For both species, only female adults and nymphs were found.

According to the morphological classification, six *T. cavicola* and six *T. neglectus* were collected and pro-

cessed for molecular identification (table 4). An alignment of twelve sequences of 446 base pair was obtained. Overall nucleotide frequencies were 28.1% adenine (A), 19.0% cytosine (C), 15.8% guanine (G), and 37.1% thymine (T). The comparison with BOLD sequences (Ratnasingham and Hebert, 2007) and the NJ tree (figure 4) confirmed the identification obtained with morphological characters for all specimens. Samples 2192c1a, 2192c1b, 2668c1, 8040c2, 2052c2, and 8028c2 belong to *T. cavicola*, while samples 2192n1a, 2192n1b, 2556n1, 5013n2, 5014n2a, and 5014n2b belong to *T. neglectus*. The two species are well separated and each one is well supported, with a bootstrap value of 100 for both *T. cavicola* and *T. neglectus*. Considering only the specimens collected during the present work, the mean p-distance \pm SEM within group was 0.000 ± 0.000 for *T. cavicola* and 0.003 ± 0.002 for *T. neglectus*, while the mean p-distance \pm SEM between groups was 0.132 ± 0.054 .

Discussion

Records of the presence of *Troglophilus* populations in caves of the prealpine area of Lecco and Como have been available since 1958 (Capra, 1959): the only reported species belonging to this genus was *T. cavicola*,

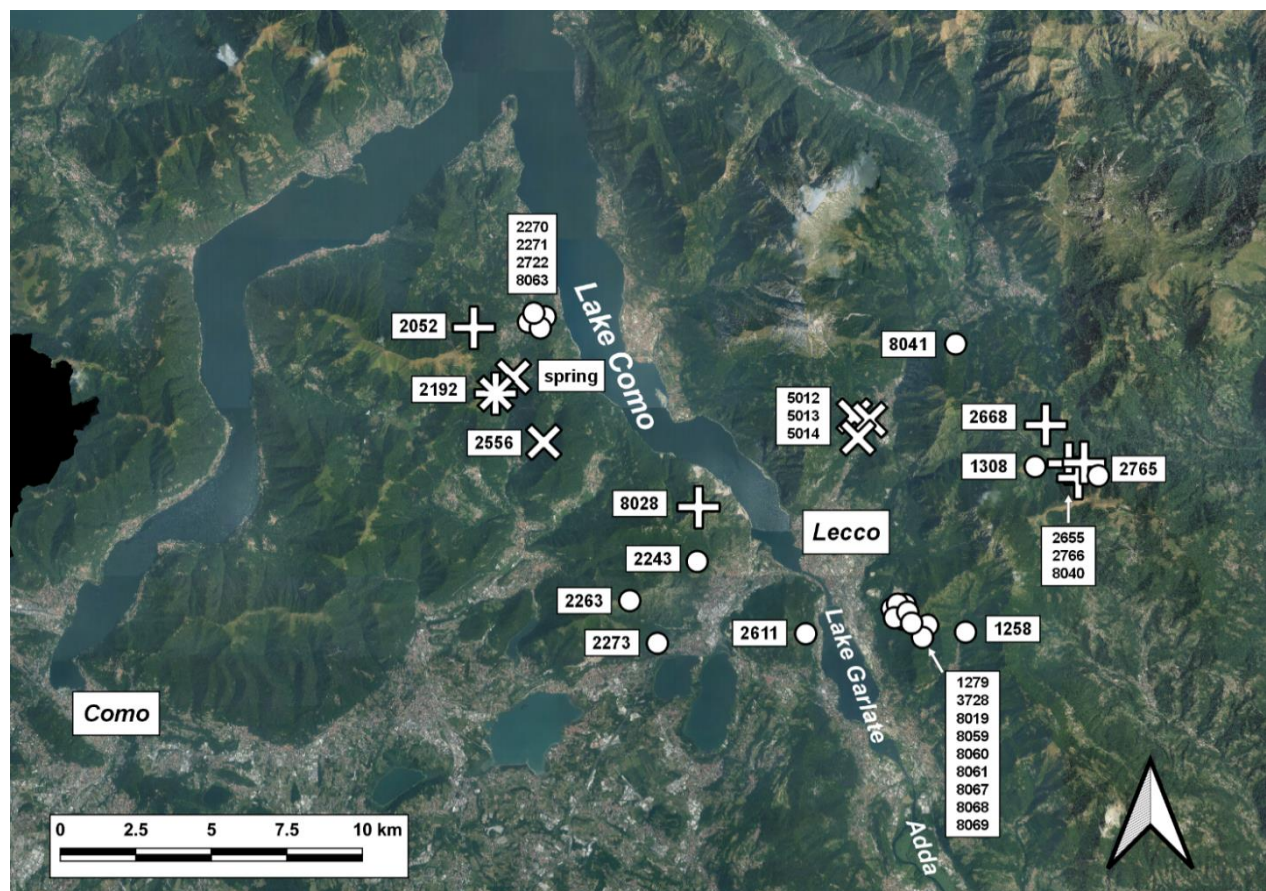


Figure 2. Results of surveys for the presence or absence of *Troglophilus*. **x** caves where *T. neglectus* was found, **+** caves where *T. cavicola* was found, ***** caves with both species, and **o** caves where no *Troglophilus* were found. Investigated area map: Map Data Google 2018.

Table 4. Results of surveys for the presence or absence of *Troglophilus*. The codes of collected specimens are composed as follows: inventory number of cave where the specimen was collected, species identification by morphology (c = *T. cavicola*; n = *T. neglectus*), and stage (1 = nymph; 2 = adult).

Group	Cave code	Cave name	Detected species	Stages	Collected specimens
a	-	Spring Catchment	<i>T. neglectus</i>	1	2192 c1a, 2192 c1b,
	LoCo 2192	Grotta del Fo' di Barni	<i>T. cavicola</i> , <i>T. neglectus</i>	1	2192 n1a, 2192 n1b
b	LoLc 2765	Primo Buchetto di Cricri	-	-	8040 c2
	LoLc 2766	Secondo Buchetto di Cricri	<i>T. cavicola</i>	2	
	LoLc 2655	Grotta nel Vallone	<i>T. cavicola</i>	2	
	LoLc 8040	Mollaci	<i>T. cavicola</i>	2	
c	LoLc 5012	Grotta di Val Naone	<i>T. neglectus</i>	1, 2	5014 n2a, 5014 n2b, 5013 n2
	LoLc 5013	Grotta del Formaggee	<i>T. neglectus</i>	1, 2	
	LoLc 5014	Grotta del Partigiano	<i>T. neglectus</i>	1, 2	
d	LoLc 2270	Buco del Palo	-	-	-
	LoLc 2271	Spaccatura a SE del Buco del Palo	-	-	
	LoLc 2722	Grotta Riparo Irene	-	-	
	LoLc 8063	Free Camping Miralago	-	-	
e	LoLc 3728	Buco di Grao	-	-	-
	LoLc 8019	Caverna di Grao	-	-	
f	LoLc 8059	Antro del Tatzelwurm	-	-	-
	LoLc 8060	Phreatichthys Aprilis	-	-	
	LoLc 8061	Edera Rock	-	-	
	LoLc 8067	Vanth	-	-	
	LoLc 8068	Charun	-	-	
	LoLc 8069	Frattura delle Ossa	-	-	
not grouped	LoLc 1258	Büsa a la Calchera dii Sing	-	-	-
	LoLc 1279	Büsun di Trécc	-	-	-
	LoLc 1308	Büs de Carigun	-	-	-
	LoCo 2052	Büs di Pegur	<i>T. cavicola</i>	1, 2	2052 c2
	LoLc 2243	Riparo della Treminoeula	-	-	-
	LoLc 2263	Grotticella del Versante Sud del Monte Rai	-	-	-
	LoLc 2273	Buco della Sabbia	-	-	-
	LoCo 2556	Büs del Negrin	<i>T. neglectus</i>	1	2556 n1
	LoLc 2611	Risorgente sopra Casa Alpe	-	-	-
	LoLc 2668	Büs di Cavrecolo	<i>T. cavicola</i>	1	2668 c1
	LoLc 8028	Grotta dei Tassi del Moregallo	<i>T. cavicola</i>	2	8028 c2
	LoLc 8041	Fessura dei Ritornanti	-	-	-

whereby, in some cases, the taxonomic identification of the individuals did not reach the species level (table 1). The aim of this work was to investigate the presence of *Troglophilus* species in caves of the aforementioned area, to confirm the presence of *T. cavicola*, and to evaluate if *T. neglectus* is also spread in the region. Moreover, another goal was to confirm the morphological identification and the goodness of the available identification keys using molecular tools.

Our results showed that not only *T. cavicola* is present in the study area, as previously reported, but also *T. neglectus* occurs. In one cave (LoCo 2192), *T. cavicola* and *T. neglectus* were found in sympatry, as observed for more eastern Italian populations by Conci and Galvagni (1943) and Baccetti and Capra (1969). In 6 caves only *T. cavicola* was found, in 5 caves only *T. neglectus*,

and in 21 caves neither of the two species. As indicated by our very preliminary presence-absence data, the species are not present in all studied caves of the area.

The investigation of *Troglophilus* species presence in the study area was supported, for the first time, by molecular analyses. An effective, inexpensive, and rapid protocol was adapted for *Troglophilus* DNA extraction, starting from the CTAB protocol proposed by Doyle and Doyle (1987). The importance of a standardized and efficient protocol can be very useful when working with a large number of samples. The genomic DNA obtained was of good quality for the subsequent amplification of COI. Moreover, the molecular identification through COI barcoding confirmed the effectiveness of the identification keys used (Baccetti and Capra, 1969; Karaman *et al.*, 2011) for both adults and nymphs, as morpholog-

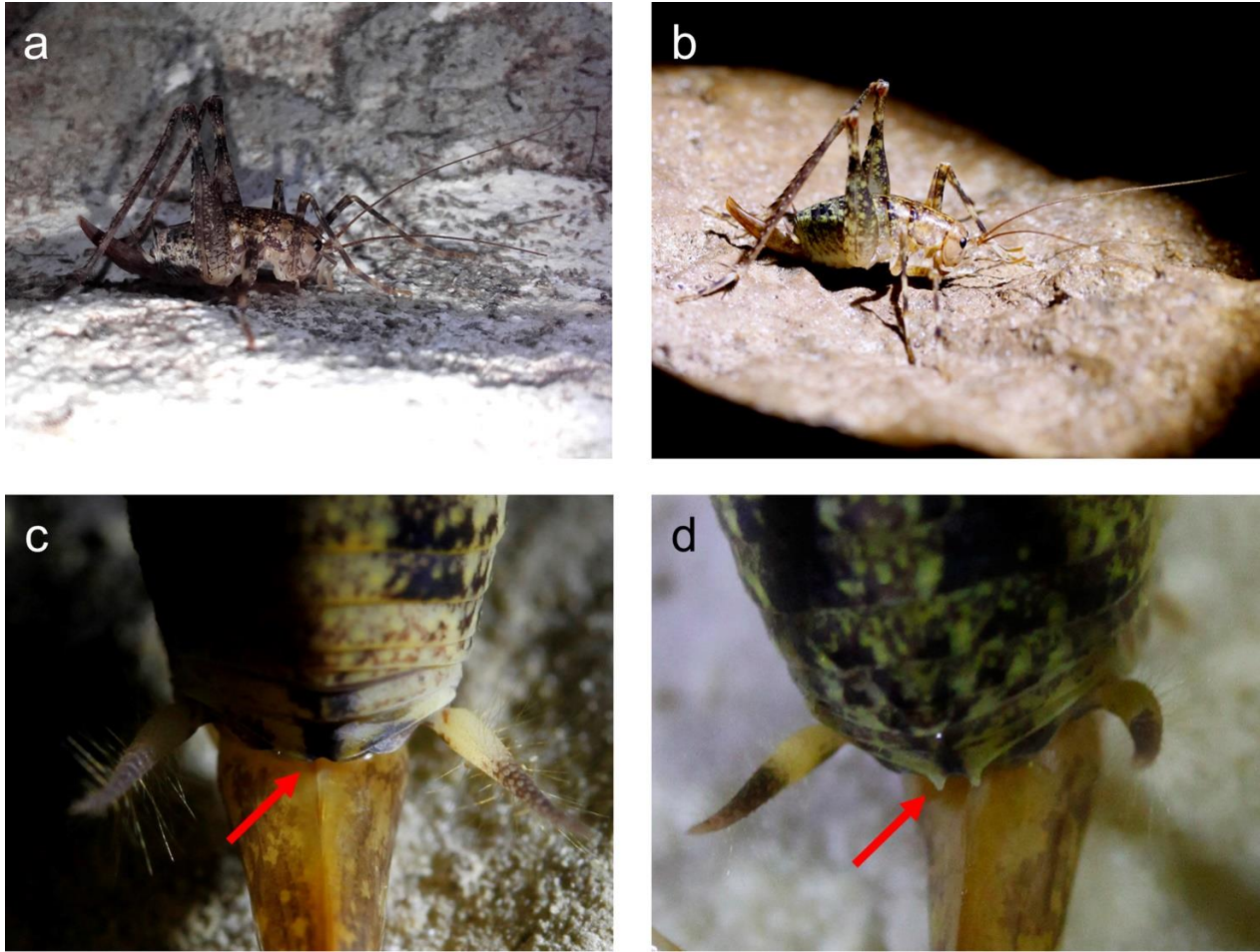


Figure 3. Detected species of *Troglophilus*. *T. cavicola* (a = adult; c = abdominal apex, dorsal view) and *T. neglectus* (b = adult; d = abdominal apex, dorsal view), female individuals. The arrows indicate tenth tergite.

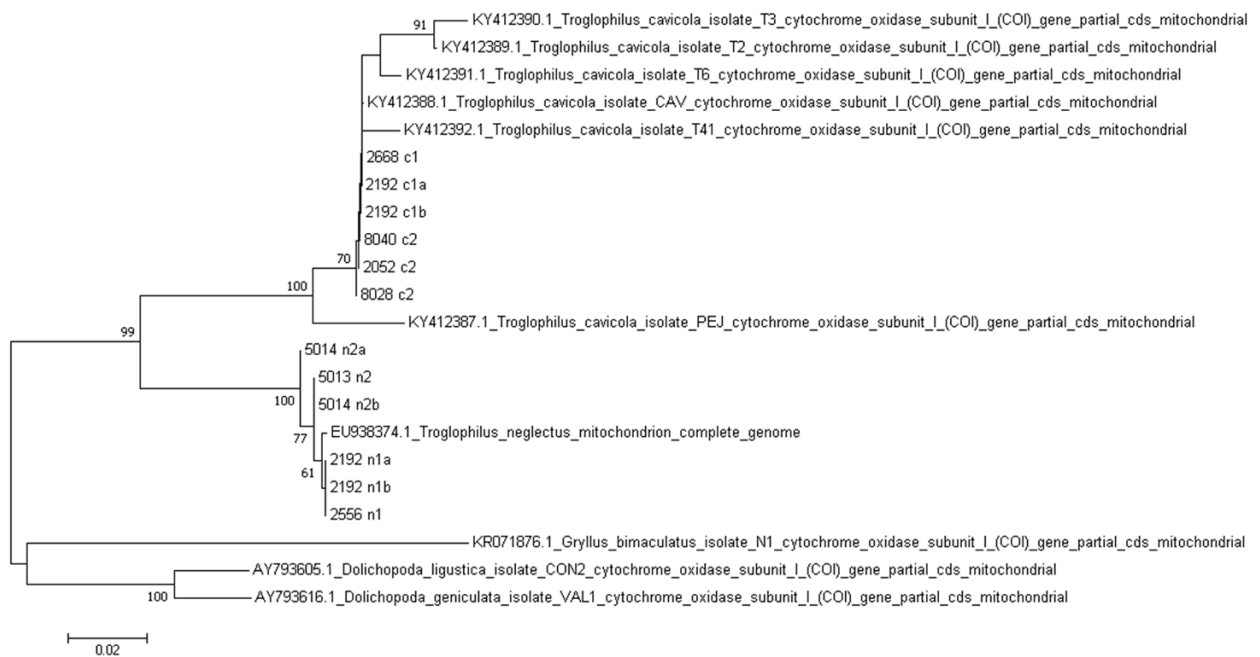


Figure 4. Neighbour-Joining tree based on genetic p-distances of COI barcode sequences of *Troglophilus* species. Numbers near nodes indicate bootstrap values (>50%). The distance scale bar is given.

ical and molecular identifications were always concordant. The creation and development of a rich DNA barcoding library is essential for identifying specimens when experienced taxonomists are not available for morphological identification.

All the sexually recognizable individuals detected during the present study were females, for both species. Our findings confirm the hypothesis of geographic parthenogenesis of *T. cavicola* in Lombardy (Capra, 1951; Baccetti, 1961; Banti *et al.*, 1981); moreover, the presence of parthenogenetic populations was also assessed for *T. neglectus*, without detecting any males for this species either. Geographic parthenogenesis is a well-known pattern, characterized by a higher frequency of parthenogenesis in marginal populations (e.g., Peck *et al.*, 1998; Haag and Ebert 2004; Pujol *et al.*, 2009; Karaman *et al.*, 2011); however, it seems that adverse climatic conditions prolonged for a long period could also favour *Troglophilus* parthenogenetic individuals and lead to the prevalence of parthenogenetic populations (Karaman *et al.*, 2011).

Regarding *T. cavicola*, it is believed that the caves in the investigated area host the westernmost marginal populations of the species range (Banti *et al.*, 1981). Regarding *T. neglectus*, the present work has provided some new information: this species was detected for the first time in the prealpine area of Lecco and Como Provinces. Thus, currently, this is the westernmost area known to host this species south of the Alps. Recently, new findings quite far from the main distribution area have been reported, in the Czech Republic (Holuša *et al.*, 1999; 2013; Chládek *et al.*, 2000), Germany (Kiefer *et al.*, 2000; Zinke, 2000), Austria (Oertel and Illich, 2011), and Switzerland (Baur and Güttinger, 2013). The origin of these populations is under debate; however, genetic analyses on two populations from Germany (Lower Saxony) and the Czech Republic revealed their similarity with Slovenian populations, arguing in favour of a recent origin, possibly anthropogenic (Ketmaier *et al.*, 2009). The discovery of new populations in the area investigated in this study, on the other hand, is very different from the aforementioned findings. First of all, the populations in Lombardy are not separated from the main ranges of *T. neglectus* by the Alps. Moreover, not only was a single population from just one or a few caves or one site reported, but rather populations from different sites, on both sides of the “water continuum” represented by Lario, Lake Garlate, and River Adda.

Taking into account that *T. neglectus* has never been found in this area before, three hypotheses could be considered for these newly discovered populations: (1) recent anthropogenic introduction of the species; (2) recent (last decades) natural spread of the species; and (3) presence but not detection of the species in the past.

The anthropogenic introduction, unintentional or deliberate, is possible. However, as reported, the populations are distributed in different caves and different sites (figure 2): a single introduction event could hardly lead to such a diffusion of the species in only few years, with a hypothetical dispersal of many kilometres through ecological barriers (the “water continuum” and urbanized areas). On the other hand, it is hard to believe that

anyone would be interested in systematically introducing this species into different areas. Finally, more than one unintentional event of introduction could offer a possible explanation, although it remains to be demonstrated how such an event could occur.

Since previous studies on *Troglophilus* did not report the presence of *T. neglectus* in Triangolo Lariano (Capra, 1959; Comotti, 1986; Banti *et al.*, 1981; 1991), a natural spread of this species toward the west could have occurred in the last few decades. However, a theory involving a recent range expansion from east toward west, possibly favoured by recent ecological and climatic changes, has many weaknesses. First, although these insects are also active on epigeal habitats, surface dispersal is assumed to be not very rapid, given the ecological characteristics of the species (Ketmaier *et al.*, 2009). Moreover, we reported the presence of *T. neglectus* in caves 50 kilometres from the westernmost population known in Lombardy before the present work, the “Buco del Corno” cave (LoBg 1004). It seems impossible that the species could, in a few years, spread tens of kilometres crossing rivers and ecological barriers.

The third hypothesis assumes that *T. neglectus* was already present in the investigated area in the past but was never detected. There is a lack of knowledge about long-term population fluctuations of *Troglophilus* species, but fluctuations were observed in different years and seasons (Pehani *et al.*, 1997; Di Russo *et al.*, 2008). It could be possible that in past decades, when most of the studies of *Troglophilus* distribution in Lombardy were conducted, the population level of *T. neglectus* was lower in this area than it currently is, and that the presence of a few individuals of this species may not have been noticed. Moreover, information about seasonal population fluctuations was not available in the past and it is possible that some surveys were conducted during summer, season in which the population levels are lower (Pehani *et al.*, 1997; Lipovšek *et al.*, 2011; 2016; Di Russo *et al.*, 2014). Finally, in the past no molecular tools were available to easily determine the specimens of uncertain identification.

The present remarks should be considered as hypotheses on which to base further research.

Moreover, this paper provides useful distributional data for both investigated species, providing information that must be considered to understand the biogeographic history of these species as accurately determining their range borders represents a crucial prerequisite for addressing this question (Zeisset and Beebe, 2001).

In particular, the recent distribution of *T. cavicola* and *T. neglectus* out of the Balkans, in the west, is considered to be shaped by their postglacial expansion (Karaman *et al.*, 2011), and it has been hypothesized that both species probably reached their current distribution area in Northern Italy sometime in the Quaternary, when climatic conditions became favourable (Ketmaier *et al.*, 2004). However, currently, no molecular data are available to confirm this theory. To obtain reliable results, a population structure analysis and biogeographic studies, including mitochondrial and nuclear sequences from all distribution range of these species, should be performed. Indeed, molecular data from present populations form

the basis to reconstruct the historical process of the recolonization pathway, location of refugia, and demographic bottlenecks or expansion (Avisé, 2000).

The updated confirmation of *T. cavicola* presence and the discovery of the widespread occurrence of *T. neglectus* in the study area must be considered for any sampling plan to address biogeographic issues, also taking into account that Triangolo Lariano has a very peculiar climatological history. Indeed, a large part of this area was affected by the Quaternary glaciations, with few areas not directly influenced by the extreme climatic conditions (Bini *et al.*, 1998; 2009) that might have represented favourable refugia for organisms.

In our opinion, a multidisciplinary approach, considering molecular, distributional, historical, paleoclimatological, and ecological data is mandatory in order to explain how the present distribution of these species was shaped by the impact of Quaternary climatic-ecological fluctuations and by the possible presence of glacial refugia, but also taking into account the present-day ecological conditions and a possible anthropic intervention.

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Authors' addresses: Marco BONELLI (corresponding author: marco.bonelli@unimi.it), Department of Biosciences, University of Milan, via Celoria 26, 20133 Milan, Italy; Silvia MESSINETTI, Department of Environmental Science and Policy, University of Milan, via Celoria 2, 20133 Milan, Italy; Felicita SPREAFICO, Department of Earth and Environmental Sciences, University of Milano-Bicocca, piazza della Scienza 4, 20126 Milan, Italy.

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