Organisms Diversity & Evolution
https://doi.org/10.1007/s13127-024-00657-8

ORIGINAL ARTICLE

Lights on tardigrade biodiversity: integrative redescription of *Eremobiotus alicatai* (Eutardigrada, Isohypsibiidae) with new insights on its morphology, phylogeny, and biogeography

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Received: 25 June 2024 / Accepted: 19 September 2024

Abstract
Current knowledge on the genus *Eremobiotus* still remains limited. Only three species are known, mainly recorded in the Palaearctic region, with *Eremobiotus alicatai* representing the most common species. In the present study, an integrative re-description of *E. alicatai* based on the examination of a topotypic population from Sicily (Italy) is given, the morphological characters of which perfectly correspond to those of the type series collected in 1969. A second population of *E. alicatai*, from Tuscany (Italy), was also investigated from the morphological and the molecular point of view, allowing examining the intraspecific variability of the species. A re-analysis of the morphology of specimens of the type series, along with a morphological analysis of specimens of the two investigated populations of *E. alicatai*, allowed for an update and correction of certain morphological traits in the species. The topotypic and the Tuscanian populations were both investigated through a molecular approach: COI, ITS2, 18S, and 28S gene sequences were obtained, allowing to update the Isohypsibioidea phylogeny and to discuss the correct placement of the genus *Eremobiotus*. Moreover, the definition of the morphology of the claws of *Eremobiotus*-type and new observations regarding the buccal opening are discussed.

Keywords Isohypsibioidea · Morphology · Integrative taxonomy · Phylogeny · Xerophilic species · Biogeography

Introduction

Tardigrades are aquatic micro-metazoans with dimensions ranging from about 50 to 1200 µm. They inhabit almost all marine and limno-terrestrial environments, forming part of the meiofaunal communities that include other microscopic animals such as rotifers, nematodes, and gastrotrichs. Currently, more than 1460 species of tardigrades are known (Degma & Guidetti, 2007, 2023; Guidetti & Bertolani, 2005), and many of them, described in the past and of which there is lack of data, continue to pose challenges for correct specific diagnosis.

A few decades ago, the description of a tardigrade species new to science could involve exclusively detailed drawings of the diagnostic structures of the animals and their eggs. Nowadays, with the improvements in study methodologies of these organisms, a species description solely relying on illustrations of diagnostic characters is no longer sufficient. It has become necessary to employ modern research tools such as high-resolution cameras mounted on phase-contrast (PCM) or differential interference contrast (DIC) microscopes and, if possible, scanning electron microscope (SEM). Moreover, after a thorough morphological study, a more accurate definition of the species can be obtained by integrating its molecular characterization using mitochondrial (e.g., COI) and nuclear markers (e.g., 18S, 28S, ITS2).

So far, a significant number of tardigrade species, described in the past with methods today outdated, have been re-described (e.g., Zawierucha et al., 2016, Kaczmarek et al., 2018, 2022, Gąsiorek et al., 2018, Guidetti et al., 2019, Stec et al., 2018, 2020a, Tumanov, 2020, Mioduchowska et al., 2021, Grobys et al., 2020). This represents a crucial

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Published online: 23 October 2024

turning point in gaining a clearer knowledge of species, or groups of species, that previously were placed in a state of disarray. As a matter of fact, modern re-descriptions can contribute to better understanding the morphology and phylogeny of the studied taxon.

One of the most debated tardigrade superfamilies is Isohypsibioidea Sands et al., 2008, established based on molecular data obtained through 18S analysis (Sands et al., 2008). This superfamily currently comprises five families: Doryphoribiidae Gąsiorek et al., 2019a; Halobiotidae Gąsiorek et al., 2019a; Hexapodibiidae Cesari et al., 2016; Ramajendidae Tumanov, 2021; and Isohypsibiidae Sands et al., 2008. In particular, the latter includes 8 genera and 103 species and still encompasses lineages that require further analysis (Bertolani et al., 2014; Gąsiorek et al., 2019a; Guil & Giribet, 2012; Mioduchowska et al., 2021); one of these lineages is represented by the genus *Eremobiotus* Biserov, 1992, whose phylogenetic position within the Isohypsibiidae has remained uncertain and in need of further investigations (Mioduchowska et al., 2021).

In the present work, the species *Eremobiotus alicatai* (Binda, 1969) was redescribed through a modern approach of integrative taxonomy of two populations of the species, one from its *locus typicus* (Gela, Sicily, Italy) and the other from Orbetello (Grosseto, Tuscany, Italy). The majority of the paratypes of *E. alicatai* are in poor condition and useless for morphological analysis. For this reason, we also must stress the importance of redescribing the species. The present study also allowed to amend the description of *Eremobiotus ginevrae* Lisi et al., 2016, and to update the phylogenetic position and geographic distribution of *Eremobiotus* species. Furthermore, some typical morphological characters of the genus, particularly regarding peribuccal structures and claws, proved to need amendments and clarifications.

Background knowledge on the genus *Eremobiotus*

Eremobiotus currently comprises only three species, i.e., *E. alicatai*, *E. ginevrae*, and *Eremobiotus ovezovae* Biserov, 1992. They can be typically found in soil habitats, as supported by the claw morphology and leg structures (Bertolani, 1983; Bertolani & Biserov, 1996). The genus was erected by Biserov (1992), who defined it on the presence of six peribuccal papulae and claws of modified *Isohypsibius* type, which show a peculiar morphology due to the angle formed between primary and secondary branch, which is about 180° in internal claws of legs I–III and in both anterior and posterior claws of legs IV.

Gąsiorek et al. (2019a) amended the genus description by reporting a new morphological character, i.e., the first band of teeth in the oral cavity armature (OCA) system, which comprises two to five rows of medium-sized and densely arranged conical teeth.

Claws and peribuccal structures of *Eremobiotus* species have been mentioned by Pilato and Binda (2010) and Gąsiorek et al. (2019a); however, no definitive conclusion has been reached that could encompass all species within the genus, thereby rendering the diagnostic characters of the genus unclear. In particular, (i) the angle formed by the two branches of claws I–III with each other remains ambiguous, with various definitions proposed over the years (Biserov, 1992; Gąsiorek et al., 2019a; Pilato & Binda, 2010), and (ii) claws IV, for which the definition does not adequately account for the inter- and intraspecific variability among animals belonging to the known species.

We therefore intended to discuss those problems (i.e., shape and structure of claws and peribuccal structures), through the revision of the genus morphological characters, and the analysis with light microscopy (LM) of type material of *E. alicatai*, *E. ginevrae*, and *E. ovezovae* and through LM and SEM investigations of two populations of *E. alicatai*; in this way, we were able to amend and update the genus definition.

Eremobiotus distribution

The distribution of the species of the genus is predominantly Palearctic, with only one record in North America (Johansson et al., 2011) (Fig. 1). *Eremobiotus* species are distributed in arid environments, often halophilic; they may have a broader distribution than currently known, as these environments have been underexplored over the years. The highest number of records regards Italy (Bertolani, 1975, 1983; Binda, 1969; Binda & Pilato, 1972; Lisi et al., 2016) and Poland (Dastych, 1988) (Fig. 1). The main distribution area, i.e., central Europe, may be biased due to a lack of data from other countries. All the data regarding points indicated in the Fig. 1 are reported in Table SM.01. The report of the genus from freshwater (Pilato, 1973), not depicted in the Fig. 1, should be verified through an analysis of new material before to be added to the genus distribution.

Typical environment of *Eremobiotus alicatai*

The species *Eremobiotus alicatai* was described from a moss sample collected in a peculiar Sicilian environment, i.e., the coastal sandy dunes near Gela, an extensive complex of dunes ca. 62 km along the Gulf of Gela (Figs. 1 and 2). Given their significant naturalistic value, these dune systems are subject to a range of conservation measures, being classified as a Natural Reserve, Site of Community Importance (SCI), Special Protection Area (SPA), and Important Bird Areas (IBA) (Sciandrello et al., 2015). However, the coastal dunes of the Mediterranean area are among the most vulnerable and seriously threatened ecosystems due to human activities; this is also confirmed by the sandy dune environments located near Gela (Sicily, Italy), which showed a large spatial reduction since 1938 (Sciandrello et al., 2015).

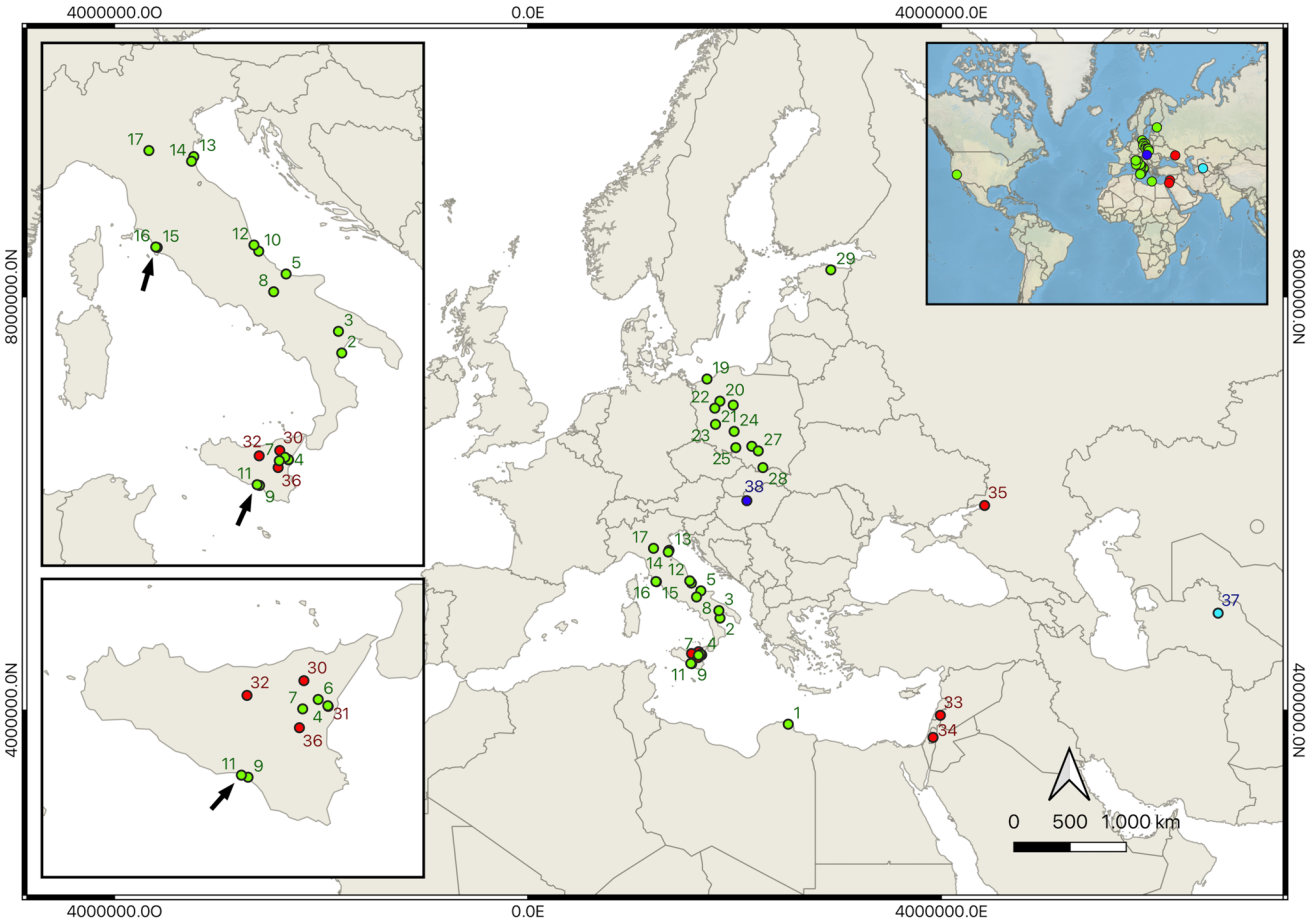


Fig. 1 Maps showing all the reports for *Eremobiotus* species, with the main distribution areas located in central Europe. Green: *E. alicatai*; red: *E. ginevrae*; light blue: *E. ovezovae*; dark blue: *Eremobiotus* sp. Arrows indicate the localities involved in the present study (11: Gela, fossil dunes; 16: Orbetello, Fonteblanda). All the data regarding the reports, including the report for North America as reported in the miniature in the upper right angle of the figure, are shown in Table SM.01

Eremobiotus alicatai was recorded for the first time in 1969 (Binda, 1969; "in muschi che si sviluppano sulle dune costiere fossili di Gela," i.e., in mosses developing on the fossil coastal dunes of Gela) and proved to be strictly linked to this environment, being found again (in this study) in the same locality and moss species after about 55 years, despite the intense human activity. In particular, the species was found in a sample of moss on sand belonging to the species *Tortella flavovirens* (Bruch) Broth., a psammophilous moss species that has a key role in fixing the sand and so promoting sand stability and soil development (Murru et al., 2018; Warming, 1909).

Material and methods

Sampling

A sample (University of Catania sample code Unict-DFG2) of *T. flavovirens* moss on sand was collected in the *locus typicus* of *E. alicatai* (Fig. 2). The sample was collected on November 6, 2022, at 37°05′32″ N 14°10′06″ E, 5 m asl (Fig. 1). The sample was immediately transferred to the laboratory. A portion of the sample was placed in tap water for 20 min and subsequently washed using two sieves having mesh dimensions of 250 µm and 37 µm; the remaining sample was air dried at c.a. 20 °C and preserved at room temperature for future studies. The material restrained by the 37 µm sieve was observed with a stereomicroscope, and tardigrades were extracted using a Pasteur pipette.

A sample (University of Modena and Reggio Emilia sample code C3089) of turf close to the shore was collected on December 21, 2009 (Bertolani leg.) in Fonteblanda (Orbetello, Grosseto, Tuscany, Italy), at 42°32′40″ N, 11°10′30″ E, 6 m asl (Fig. 1). The sample was put in a Baermann funnel, and the material collected from the funnel was sieved with a 37 µm sieve and observed with a stereomicroscope. Tardigrades were extracted using a Pasteur pipette.

Fig. 2 *Locus typicus* of *Eremobiotus alicatai*. **a** Sandy environment with sparse mosses on sand. **b** Detail of the moss identified as *Tortella flavovirens*

Morphometric analysis

Animal structures were measured only if their orientation was suitable. Body length was measured from the buccal opening to the end of the body, excluding the hind legs. Buccal tube length and the level of the stylet support insertion point were measured according to Pilato (1981). Buccal tube width was measured as the external and internal diameter at the level of the stylet support insertion point. Claws I–III were measured according to Beasley et al. (2008). Claws IV, due to their unusual shape and structure (the latter also preventing from clearly see internal septa), were measured with a new method presented in the "Results" section. The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage (Pilato, 1981). Macroplacoid length sequence is given according to Kaczmarek et al., 2014. Morphometric data were handled using the "Parachela" template available from the Tardigrada Register (www.tardigrada.net/register, Michalczyk & Kaczmarek, 2013).

Molecular and phylogenetic analysis

Total genomic DNA was extracted from single adult tardigrades (Table 1). The extractions were carried out using QuickExtract™ DNA Extraction Solution (Lucigen, Middleton, WI, USA), following the manufacturer's instructions. All animals were previously observed *a fresco* in a drop of water with LM up to 100× in order to collect pictures before DNA extraction according to Cesari et al. (2011). Four DNA fragments were amplified: the small ribosome subunit 18S rRNA, the large

Table 1 List of tardigrade specimens utilized for molecular analysis and GenBank accession numbers. In bold sequences produced in this study.

Specimen	Locality	18S	28S	ITS2	COI
C5043 V1	Gela (Sicily, Italy)	**PQ386447**	**PQ386460-PQ386453**	**PQ428930**	N/A
C5043 V2	Gela (Sicily, Italy)	**PQ386448**	**PQ386461-PQ386454**	**PQ428931**	**PQ386470**
C5043 V3	Gela (Sicily, Italy)	**PQ386449**	**PQ386462-PQ386455**	**PQ428932**	**PQ386471**
C5043 V4	Gela (Sicily, Italy)	**PQ386450**	**PQ386463**	**PQ428933**	**PQ386472**
C3089 V1	Orbetello (Tuscany, Italy)	N/A	KT778608	N/A	**PQ386466**
C3089 V2	Orbetello (Tuscany, Italy)	N/A	N/A	**PQ428928**	**PQ386467**
C3089 V3	Orbetello (Tuscany, Italy)	**PQ386446**	N/A	**PQ428929**	**PQ386468**
C3089 V7	Orbetello (Tuscany, Italy)	N/A	N/A	N/A	**PQ386469**
C3089 Ersp A1	Orbetello (Tuscany, Italy)	HQ604951	N/A	N/A	N/A
C3089 Ersp A2	Orbetello (Tuscany, Italy)	HQ604952	N/A	**PQ428926**	N/A
C3089 Ersp A3	Orbetello (Tuscany, Italy)	HQ604953	N/A	**PQ428927**	N/A

N/A not available

ribosome subunit 28S rRNA, the internal transcribed spacer ITS2, and the cytochrome oxidase subunit I COI, with the primers and protocols described in Giribet et al. (1996), Whiting et al. (1997), and Bertolani et al. (2014) for the 18S gene; in Schwendinger and Giribet (2005), Mironov et al. (2012), and Gąsiorek et al. (2019b) for the 28S gene; in Wełnicz et al. (2011) for the ITS2 marker; and in Folmer et al. (1994) and Prendini and Wheeler (2005) for the COI gene. The amplified products were purified from the gel by using the Wizard Gel and PCR cleaning (Promega) kit, while sequencing reactions were performed as described in Cesari et al. (2022). Nucleotide sequences of the newly examined specimens were deposited in GenBank (accession numbers: PQ386466-72 for the COI gene; PQ428926-33 for the ITS2 gene; PQ386453-5 and PQ386460-2 for the 28S gene and PQ386446-50 for the 18S gene; Table 1).

Phylogenetic analysis was conducted on the 18S and 28S genes. Sequences were aligned using the MAFFT algorithm (Katoh et al., 2002) accessed through the MAFFT online service (Katoh et al., 2017) and verified through visual inspection. Sequences from Eohypsibioidea Bertolani & Kristensen, 1987; Macrobiotoidea Thulin, 1928; and Hypsibioidea, Pilato, 1969a were employed as an outgroup, and available Isohypsibioidea sequences from GenBank were incorporated into the analysis (Table SM.02). Phylogenetic analysis was conducted using both maximum likelihood (ML) and Bayesian inference (BI) methods, via the CIPRES platform. The evolutionary model for the 18S and 28S genes was determined using the corrected Akaike information criterion within jModeltest 2.1.10 (Darriba et al., 2012; Guindon & Gascuel, 2003) resulting in the selection of the GTR + G model. The ML tree was computed using RAxML version 8.2.12 (Stamatakis, 2014). Bootstrap resampling comprising 1000 replicates was performed using the rapid bootstrap procedure proposed by Stamatakis et al. (2008) to assess branch support in the ML tree. The BI dendrogram was computed with the MrBayes software (Ronquist et al., 2012) v.3.2.7a. Two independent runs, each of four Metropolis coupled Markov chains Monte Carlo method, were launched for 6×10^7 generations, and trees were sampled every 1000 generations. Convergence of runs and making sure that its value was < 0.005 assessed by tracking the average standard deviation of split frequencies between runs and by plotting the log likelihood of sampled trees in Tracer v.1.7 (Rambaut et al., 2018). The first 6×10^6 sampled generations were discarded as burn-in.

For the analysis of species delimitation, sequences of COI and ITS2 markers were utilized. Nucleotide sequence divergences among identified haplotypes were computed using the p-distance method with MEGAX (Kumar et al., 2018). Species delimitation analyses were conducted using the Assemble Species by Automatic Partitioning (ASAP; Puillandre et al., 2021) method and the Poisson Tree Process (PTP; Zhang et al., 2013). The distance-based ASAP analysis was carried out on both markers using the ASAP website (https://bioinfo.mnhn.fr/abi/public/asap/, accessed on 15-VI-2024). The PTP analysis was performed on the COI gene only using an initial ML gene tree computed with RAxML via CIPRES, employing the GTR + G model selected as described above. A sequence of *Ramazzottius varieornatus* Bertolani & Kinchin, 1993 (Eutardigrada, Macrobiotoidea; GenBank acc. no.: NC_031407) was used as the outgroup. Bootstrap resampling (1000 replicates) was conducted as previously described. Moreover, for the COI gene sequence, relationships were estimated using a haplotype parsimony network based on the approach outlined by Templeton et al. (1992), implemented in TCS 1.21 (Clement et al., 2000) and visualized using tcsBU (Santos et al., 2016). A 95% connection limit was applied (Hart & Sunday, 2007).

Scanning electron microscopy analysis

Three *E. alicatai* specimens from the *locus typicus* were prepared for SEM analysis following the "A2" protocol described in Camarda et al. (2023), sputter coated with gold and observed with Phenom-XL G2 SEM.

Light microscopy analysis

The holotype and 9 paratypes of *E. alicatai* from the Binda and Pilato collection (University of Catania, Italy), additional 20 specimens of *E. alicatai* newly extracted from the re-sampled moss in the *locus typicus*, mounted in Polyvinyl Lactophenol, and 11 specimens of *E. alicatai* collected in Orbetello (Tuscany, Italy), mounted in Faure liquid (Slide Nos. C3089 S1a_6062–S9_6068) and Hoyer's mounting media (Slide Nos. C3089 V1_6069–V6_6072) were observed with PCM Leica DM1000. Photos were taken with the digital camera Flexacam C3.

The holotype and eight paratypes of the species *Eremobiotus ginevrae* and one paratype of *Eremobiotus ovezovae*, from the Binda and Pilato collection (University of Catania, Italy), were also observed for an updated differential diagnosis.

Figures and map assembly

Photographic material obtained with PCM and SEM was enhanced (contrast, light adjustments, background removal) and assembled in tables using GIMP (version 2.10). The map in Fig. 1 was created with Q-GIS (version 3.40 Madeira).

Results and discussion

Peribuccal papulae definition

In eutardigrades, peribuccal papulae could be identified as small, round, and not clearly lamellar peribuccal structures

around or on the mouth opening; the option "around or on" reflects the fact that, as results from the literature, non-homologous structures were brought together under the name "papulae" in different genera; another problem is that, sometimes, there could be confusion with peribuccal lobes (circumoral sensory field); thus, we propose to use the following nomenclature and definitions of papular-shaped structures (i.e., round peribuccal structures, as also stated by Gąsiorek et al., 2019a), in order to distinguish between two different types, mainly based on the position and consequent homology:

- Around the mouth opening, as observable in *Calohypsibius ornatus* (Richters, 1900) in Gąsiorek et al. (2019a); those structures, according to the position with respect to the oral cavity, should be homologous to peribuccal lobes. We will refer to those structures as "papular peribuccal lobes".
- On the buccal ring structure, as observable in *Minibiotus* R.O. Schuster, 1980 (in Schuster et al., 1980) (typical trait of the genus, also discussed by Stec et al., 2020b); those have to be considered homologous to peribuccal lamellae due to their position (on the buccal ring margin), number (they are 10 as the peribuccal lamellae of the other Macrobiotoidea), and, partially, shape, as these probably are reduced, flattened lamellae (Stec et al., 2020b). In the Isohypsibioidea, those structures may have originated from the subdivision of the buccal ring in correspondence of its internal septa (septa that are well observed in *E. alicatai* and maybe present in other Isohypsibioidea species). This appears clear, for example, for the peribuccal lamellae of *Pseudobiotus* Nelson, 1980 (in Schuster et al., 1980), considered as a variable character (Gąsiorek et al., 2019a) because the buccal ring sometimes appears entire (undivided) while others is partially or totally divided into the lamellae. We will refer to all the "around the mouth" structures that are not perfectly lamellae (but considerable homologous), as "papular lamellae."

The status of Hexapodibiidae Cesari et al., 2016 requires a separate discussion. Within this family, *Haplomacrobiotus* May, 1948, exhibits a continuous buccal ring and a double system of peribuccal lobes (as observable in Fig. 1c in Cesari et al., 2016). This characteristic may be present in *Haplohexapodibius* Pilato & Beasley, 1987 also, as it is described as having "six peribuccal papulae and six peribuccal lobes." There is no mention of peribuccal papulae or lobes in the other two genera of the family (*Parhexapodibius* Pilato, 1969a, and *Hexapodibius* Pilato, 1969b), but there is lack of recent analyses (both molecular and SEM) on species belonging to these two genera; considering the challenges in observing such morphological traits and the lower attention to them in the past, it is not possible to exclude their presence, like in *Haplomacrobiotus*. If future analyses confirmed the double system of peribuccal lobes in *Haplohexapodibius*, *Parhexapodibius*, and *Hexapodibius*, this character could prove to be an apomorphy of the family Hexapodibiidae.

The issue of peribuccal papulae in *Eremobiotus*, as already mentioned in the "Introduction," is quite controversial. A drawing of peribuccal structures in Isohypsibioidea genera was also provided by Gąsiorek et al. (2019a). However, in the case of *Eremobiotus*, the illustration (Fig. 1 in Gąsiorek et al., 2019a) relied on literary sources (i.e., depicting six peribuccal papulae), and no photographs were provided to support the illustration. The presence of six peribuccal papulae represents one of the main characters of the genus *Eremobiotus* and was reported by Biserov (1992) mainly based on SEM analysis on the species *E. ovezovae*; however, in the photographs (see Fig. 3 in Biserov, 1992), the only visible structures look much more like the structures nowadays called peribuccal lobes. Given this uncertainty on this morphological peculiarity for the given species *E. ovezovae*, some hypothesis can be formulated: (i) absence of papular lamellae and presence of peribuccal lobes; (ii) presence of small papular lamellae and presence of peribuccal lobes; (iii) presence of large papular lamellae (similar to *Haplomacrobiotus* structures) and peribuccal lobes. Thus, in any case, the six peribuccal lobes are present, while there is uncertainty about the presence of papular lamellae.

In *E. alicatai*, investigations with LM apparently show the presence of papular lamellae (Fig. 3a) as it is also showed in some Isohypsibiidae species described in the past and which require further investigations (Pilato pers. comm.). However, the presence of peribuccal papulae in *Eremobiotus* is disconfirmed by SEM; as a matter of fact, six peribuccal lobes and a continuous peribuccal ring are always showed with SEM (Figs. 3b and 4a).

This situation is potentially the same as for *Dastychius* Pilato, 2013; for this genus, peribuccal papulae are mentioned by Mioduchowska et al. (2021), in the redescription of the species *Dastychius improvisus* (Dastych, 1984) suggesting that SEM analysis, given the here presented doubt about those structures observed only under LM, could clarify the morphology of its peribuccal structures, confirming or disconfirming the presence of papular lamellae.

Eremobiotus claw definition and measurement

Pilato and Binda (2010) gave a summary of all known, at that time, Eutardigrada genera; however, in the definition of the genus *Eremobiotus*, they did not mention the peribuccal papulae and defined the claws of *Eremobiotus* species of legs I–III as of "*Isohypsibius*-type"; on page 30, they wrote: "Claws of the typical *Isohypsibius* type on the first three

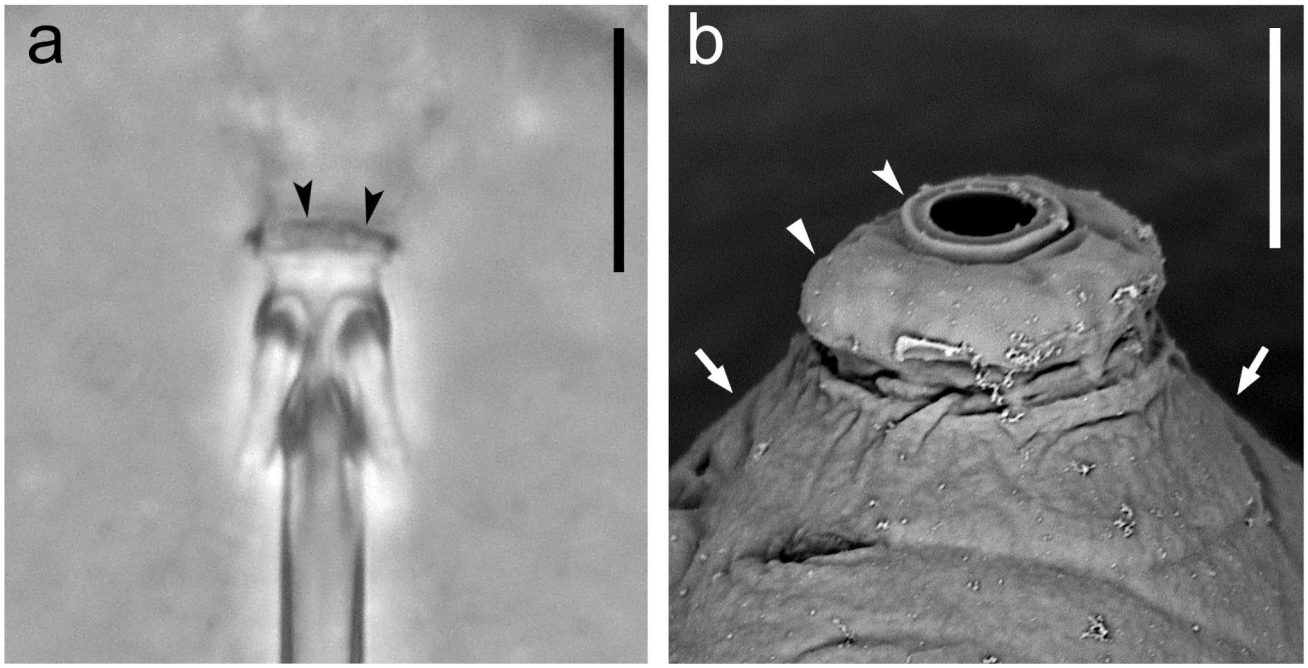


Fig. 3 Buccal opening of *Eremobiotus alicatai* (**a** PCM, topotypic population, slide no. 6046; **b** SEM, topotypic population, stub DFG2-19). **a** Buccal opening showing the subdivision of the distal buccal ring (black indented arrowhead). **b** Mouth opening with the undivided distal buccal ring (white indented arrowhead), the circumoral sensory field without evident lobes (white arrowhead) and surrounding area forming the presumed "second band of lobes" (white arrows). Scale bars **a** 10 µm; **b** 5 µm

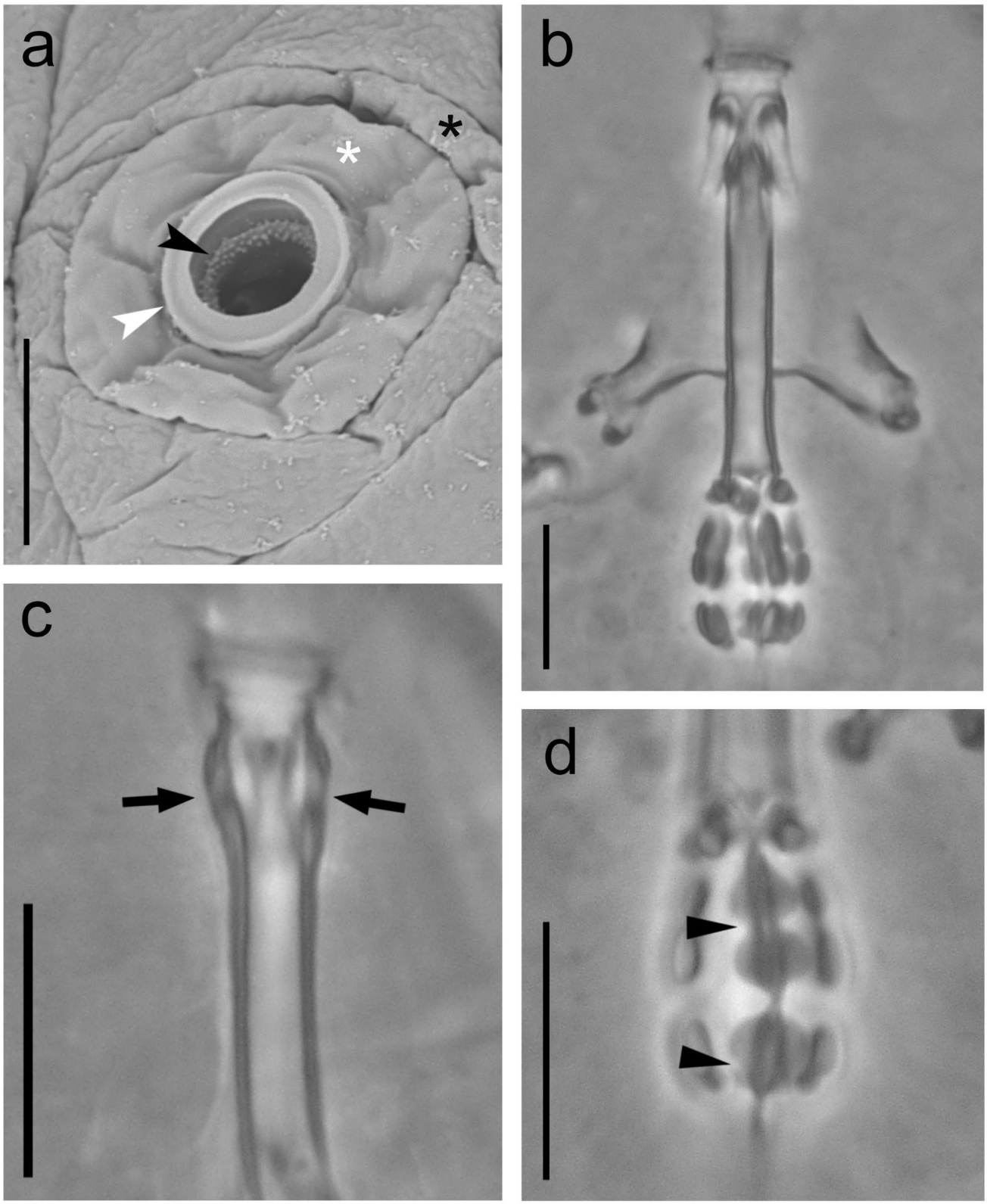
pairs of legs"; however, that definition did not take into consideration (i) the shape of internal claws I–III, in which the angle between the two branches may reach 180° and (ii) the extremely modified claws of *E. ovezovae*, obviously different from the typical *Isohypsibius* type.

Despite the significant differences (especially dimensional) between the claws of *E. ovezovae* and the other two described species, it is noticeable that in all species of the genus, the internal claws of legs I–III typically have a wider angle compared to a typical *Isohypsibius* claw (as previously reported by Biserov, 1992). However, these claws cannot be considered of the *Eremobiotus* type for various reasons (see the discussion below regarding *Eremobiotus* claws) and therefore should be considered as modified *Isohypsibius* claws (as reported by Biserov, 1992). The external claws of legs I–III appear to be of the *Isohypsibius* type.

Claws of the "*Eremobiotus* type" have never been defined formally. Biserov (1992), in describing the genus, mentioned claws of the fourth pairs of legs as "significantly different from those of I–III legs," having a large basal part of the claws, longer than that of branches, primary branches slightly longer than secondary, and an angle of approximately 180° between the two branches. Upon revising the type material of *E. alicatai*, *E. ginevrae*, and *E. ovezovae*, we observed that (i) the basal portion of the claws I–III is not always longer than the primary/secondary branch, especially in *E. ginevrae*; (ii) the claw branches of the fourth pair of legs do not always exhibit an angle of about 180°, but rather a narrower angle (approximately starting from 140°), varying even among specimens of the same population (Fig. 5d, e). In particular, the anterior claws (homologous to the internal claws of legs I–III) exhibit an angle of 160–180°, while the posterior claws (homologous to the external claws of legs I–III) display a narrower angle, of 140–180°. The branches appear to be less divergent especially in larger specimens (Fig. 5).

This variability may be dependent also on the claw orientation in the preparation. Despite this variation, the claws of the fourth pair of legs of *Eremobiotus* still differ from those of every leg pair of all Isohypsibioidea due to the following characters: (i) the primary and secondary branch share a large common portion of almost triangular shape; (ii) internal septa are almost invisible with PCM; (iii) accessory points are short, thick, and asymmetrical with respect to the main axis of the primary branch, appearing to point upwards with PCM. Thus, only those claws, i.e., the claws of the fourth pair of legs, should be treated as the "*Eremobiotus*-type" claws.

Regarding this particular claw type, some problems arise when measurements are taken with the currently used methodology of measurement of the Isohypsibioidea claws, established by Beasley et al. (2008) (see also Fig. 6a, b). Until 2002, parachelan



claws with an asymmetrical arrangement were measured according to Pilato et al. (1982), i.e., from the base of the claw to the end of the primary branch (including the accessory points), not giving troubles even if *Eremobiotus* claws were measured. Pilato et al. (2002) introduced a new method but only for the external claws of the *Hypsibius* type. Beasley et al. (2008) introduced a

◂**Fig. 4** Bucco-pharyngeal apparatus of *Eremobiotus alicatai* (specimens from the topotypic population) (**a** SEM; **b**–**d** PCM). **a** Buccal opening showing the distal buccal ring (white indented arrowhead), a single band of teeth (black indented arrowhead) and the circumoral sensory field with lobes (white asterisk) and surrounding area forming the presumed "second band of lobes" (black asterisk). **b** Dorso-ventral view of the bucco-pharyngeal apparatus. **c** Lateral view with *Isohypsibius*-type AISM (arrows). **d** Detail of the placoids; black arrowheads indicate constriction in the medial portion of the first placoids and in the final portion of the second placoid. Scale bars **a** 5 µm; **b**–**d** 10 µm

new measurement method for claws of the *Isohypsibius* type (but subsequently applied to other asymmetrical claws as well) to take into consideration also the measurement of the "basal claw height" and "secondary branch length." Up to date, this has not raised perplexities, but some difficulties are encountered if claws have primary and secondary branch diverging with an angle of approximately 180°, and no septum or flexible connection is present to clearly indicate the border that separates primary and secondary branch bases, as those of the *Eremobiotus* type. Such a situation makes it impossible to obtain an objective measurement of the structures considered.

Therefore, for those claws (*Eremobiotus* type, i.e., the fourth pair of claws, in which septa nor a clear division between primary and secondary branch is observable), we propose to not measure the claws according to Beasley et al. (2008) and re-establish the older method of claw measurement, i.e., the measurement taken from the basal portion of the claw to the end of primary branch according to Pilato et al. (1982), adding the same type of measurement also for the secondary branch (in Pilato et al. (1982) only the primary branch was measured) (Fig. 6c). Moreover, we propose a third measurement for the claws of the *Eremobiotus* type. This measurement is taken from the tip of the primary branch to the tip of the secondary branch of the same claw, excluding the accessory points (Fig. 6d). Moreover, since the "Parachela" template available from the Tardigrada Register (www.tardigrada.net/register, Michalczyk & Kaczmarek, 2013) was used, the line reporting the ratio between the posterior/anterior primary branch and the base was modified. The entry "Anterior base/primary branch (cct)" was replaced with "Anterior branch distance/primary branch (cct)," and the entry "Posterior base/primary branch (cct)" was replaced with "Posterior branch distance/primary branch (cct). This new measurement was not adopted for internal claws of the legs I–III, even if the angle sometimes reaches the 180°, due to the visibility of the insertion of the secondary branch on the primary branch, from which the measurement can be taken (Fig. 6).

Taxonomic account

Phylum: Tardigrada Doyère, 1840
Class: Eutardigrada Richters, 1926
Order: Parachela Schuster et al., 1980
Superfamily: Isohypsibioidea Sands et al., 2008
Family Isohypsibiidae Sands et al., 2008
Genus: *Eremobiotus* Biserov, 1992

Amended morphological description: Six peribuccal lobes, a buccal tube and apophyses for the insertion of the stylet muscles (AISM) of *Isohypsibius* type. Pharyngeal apophyses and two macroplacoids present in the pharynx, microplacoid, and/or septulum absent. Internal claws I–III of modified *Isohypsibius* type, forming a wide angle (about 160–180°); external claws I–III of *Isohypsibius*-type. Claws of the hind legs of *Eremobiotus* type [= with branches having an angle of 140–180°, with difficult-to-see internal septa, tendency to form a wide, triangular common tract (main body) of the claw, thick and asymmetrical accessory points], provided with indented lunulae.

Remarks

Buccal opening with apparently papular lamellae visible with PCM in all species; however, these correspond (SEM) to a continuous buccal ring with internal septa in *Eremobiotus alicatai*, and probably, but still to be verified with SEM, also in *E. ginevrae* and *E. ovezovae*.

Redescription of *Eremobiotus alicatai*

Species: *Eremobiotus alicatai* (Binda, 1969) (Figs. 3-5, 7-10).

Material examined

Holotype and paratypes of *E. alicatai*: 10 specimens; slide nos. 956, 957, 959–963, 1339, 1875, 2017.

Topotypic population of *E. alicatai*: 20 specimens; slide nos. 6023–6027, 6029, 6036, 6038–6039, 6046–6049, 6057, 6060, 6061. Three specimens; stub nos. DFG2-19, DFG2-20.

Population of *E. alicatai* from Orbetello (Grosseto): 11 specimens; slide nos. C3089 S1a_6062–V6_6072.

Type depositories: Holotype and paratypes of *E. alicatai* are preserved in the University of Catania, in the Pilato and Binda collection. Eighteen specimens of the topotypic population are preserved in the University of Catania, Pilato and Binda collection, while two are in the University of Modena and Reggio-Emilia, Bertolani collection. Specimens of the topotypic population which are mounted on SEM stub are preserved at the University of Catania (stub nos. DFG2-19, DFG2-20) in the Pilato and Binda collection. Samples of moss from which the additional specimens of *E. alicatai* were extracted are dry preserved in the University of Catania (sample UNICT-DFG2) and University of Modena and Reggio Emilia (sample C3089).

Description

Body whitish, eyespots absent. Dorsal and ventral cuticle with wrinkles (with PCM visible in exuviae and very rarely in specimens; well visible with SEM) almost invisible; cuticular

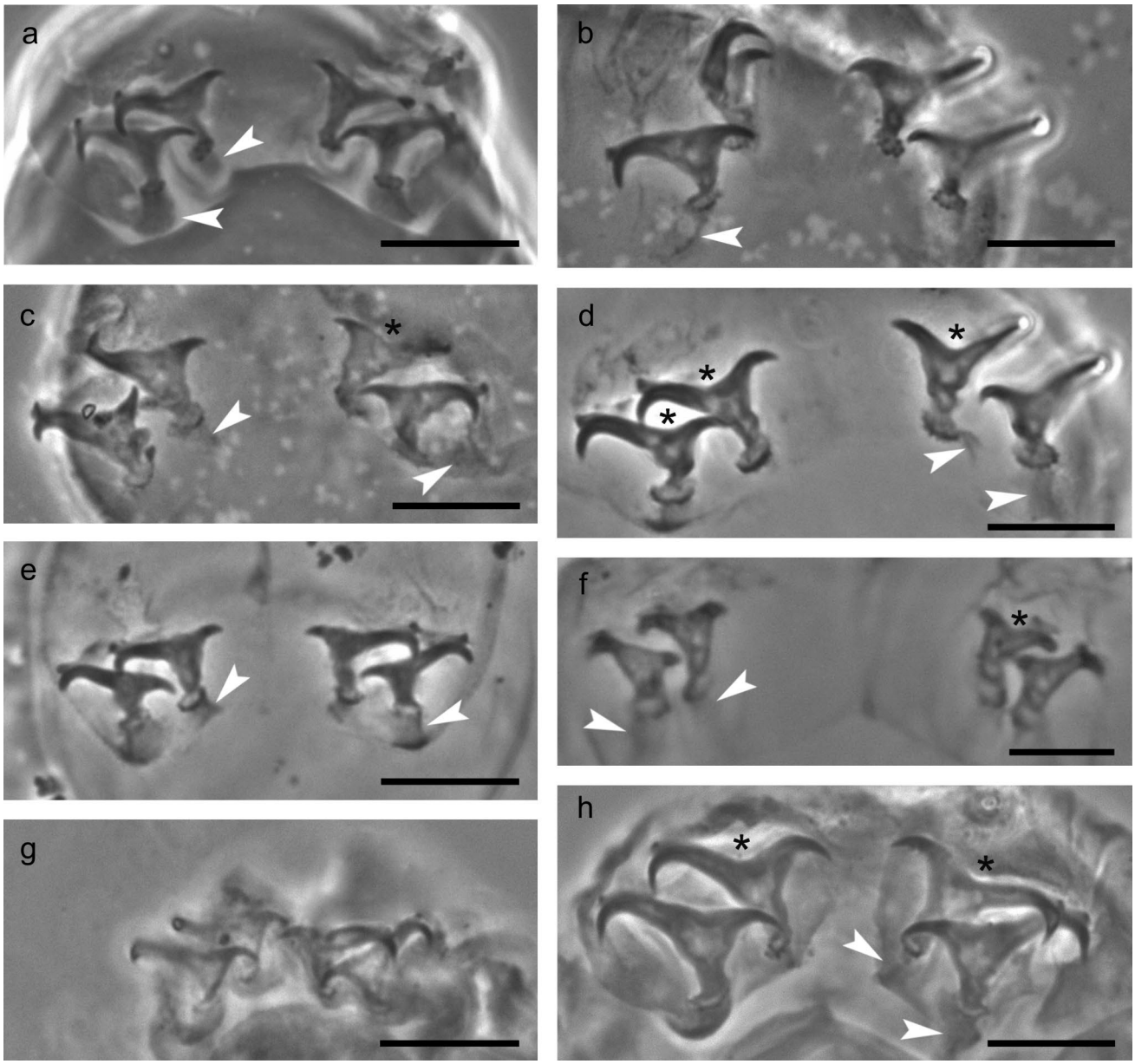


Fig. 5 *Eremobiotus* claws IV (PCM). **a**–**c** *E. alicatai,* specimens from the topotypic population; **d**, **e** *E. alicatai,* specimens from Orbetello, Tuscany population; **f** paratype of *E. ovezovae*; **g**, **h** paratype (**g**) and holotype (**h**) of *E. ginevrae*. White indented arrowheads indicate the cuticular thickening at the base of the lunula; asterisks indicate some claws with angles between primary and secondary branch visibly narrower than 180°. Scale bars: 10 µm

pores absent. Distal portion of the buccal ring with internal septa (Fig. 3a), which with PCM could give the impression of the presence of papular lamellae. Six peribuccal lobes visible with SEM (Fig. 4a, b); the oral cavity armature of *Isohypsibius* type, showing a single band with two to five rows of teeth visible only with SEM (Fig. 4a). Bucco-pharyngeal apparatus of *Isohypsibius* type; the AISM of the *Isohypsibius*-type; apophyses and two macroplacoids (length sequence 1 > 2) in the pharynx, the first of which showing a central constriction (Fig. 4b, d); microplacoid and septulum absent. Internal claws of legs I–III of modified *Isohypsibius*-type; the angle between the primary and secondary branches is very wide (ca. 160–180°) but the basal section of the claw and the insertion of the primary branch on the secondary branch still recognizable; secondary branch short, primary branch with very small and symmetrical accessory points (Fig. 7b, d); distance between the insertion point of the main branch and the claw base very short; lunules at the base of the claws present with very small teeth almost impossible to observe with PCM (Fig. 7c) but visible with SEM (Fig. 7b, d). External claws

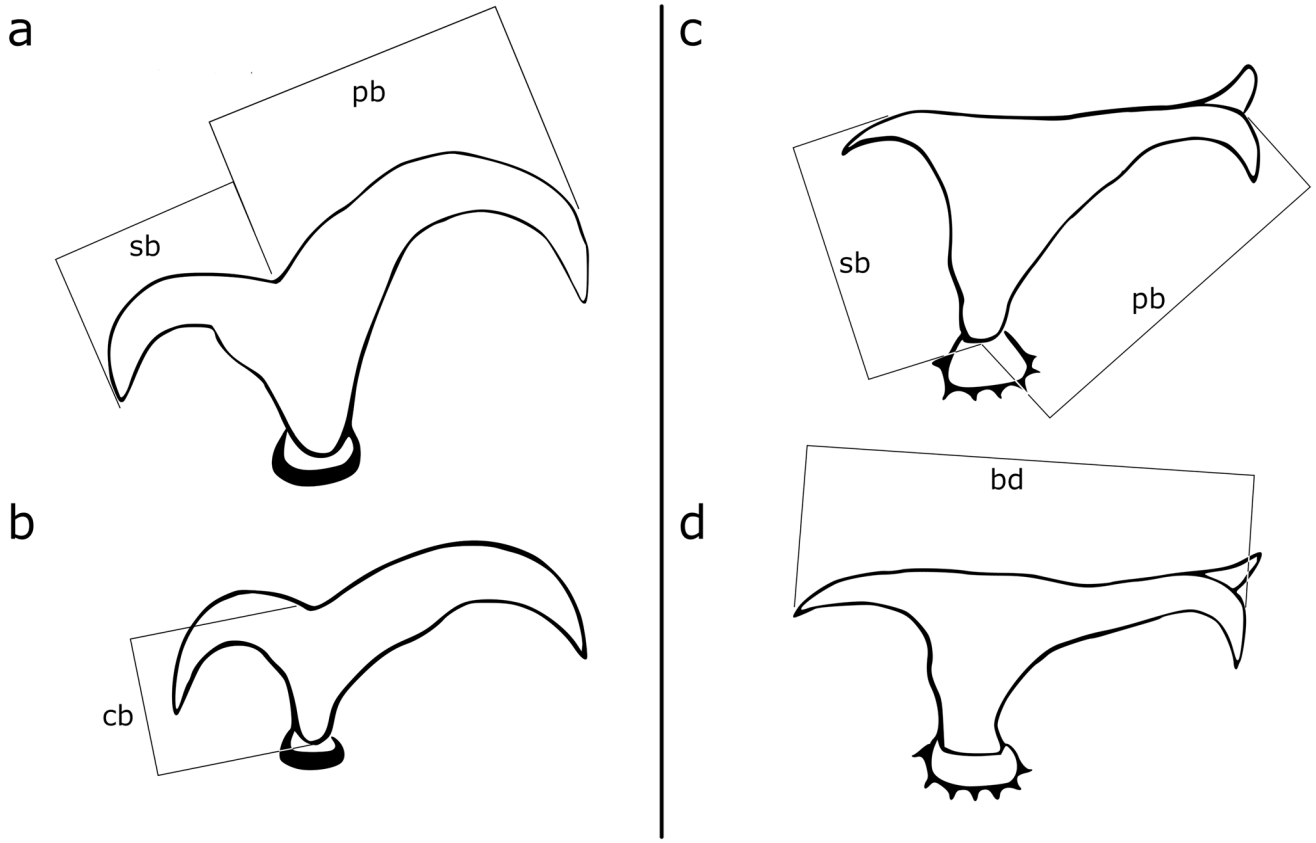


Fig. 6 Schematic drawing of the claws of *Eremobiotus alicatai* illustrating measurement methods. **a**, **b** Measurement method proposed by Beasley et al. (2008), applicable to claws I–III of *Eremobiotus* species. **a** External claw of legs I–III (*Isohypsibius*-type). **b** Internal claw of legs I–III (modified *Isohypsibius*-type). **c**, **d** Measurement method proposed here for the *Eremobiotus*-type claws (i.e., the fourth pair of claws in *Eremobiotus* species). **c** Posterior claws. **d** Anterior claws. sb, secondary branch; pb, primary branch; cb, claw base; bd, distance between branches

of legs I–III of *Isohypsibius*-type, having a lesser wide angle between primary and secondary branches (ca. 130°), secondary branch short, primary branch with very small and symmetrical accessory points (Fig. 7b, c). A long and irregular singular cuticular thickening (single indented bar) develops from the lunula of the internal claws of legs I–III (Figs. 7a, c, d and 9c), with thick and evidently indented external margins, which teeth are positioned on the external cuticle (Fig. 7d). Anterior and posterior claws IV of *Eremobiotus*-type, with primary and secondary branches fused forming a relatively long common triangular tract. The angle between primary and secondary branches very wide (ca. 170–180°); primary branches with thick and asymmetrical (with respect to the main axis of the primary branch) accessory points, giving the impression, with PCM, of a primary branch with a secondary point pointing upwards (Figs. 9d and 10); claw bases with small indented lunules which often present supernumerary small teeth (Fig. 10a–c, e, f). A cuticular thickening present at the base of the lunulae of claw IV, bigger in anterior claws and smaller and slightly visible in posterior claws (Figs. 5a–e and 10a, c). A furbelow structure present on all legs (i.e., an area surrounding the claws with fine but very dense granulation, visible with SEM (Figs. 7b, d, 8a, and 10e) and, partially with PCM (Fig. 10a, c). Eggs smooth laid in the exuviae.

Remarks

Binda (1969) reported the presence of cuticular pores ("cuticola cosparsa di perle," meaning "cuticle sprinkled with pores"); however, our analysis of types through LM, and analysis of the two investigated populations of *E. alicatai* through LM and SEM, revealed the absence of cuticular pores.

Differential diagnosis

Given that the main characters to differentiate *E. alicatai* from *E. ginevrae* are not constant and well visible in all specimens of the former species (indentation of the lunulae of the legs I–III), the two species differs only in the dimension and indentation of the lunulae of the IV pair of legs and in morphometric characters.

Eremobiotus alicatai differs from *E. ginevrae* in having bigger lunulae of the fourth pair of legs; those have a

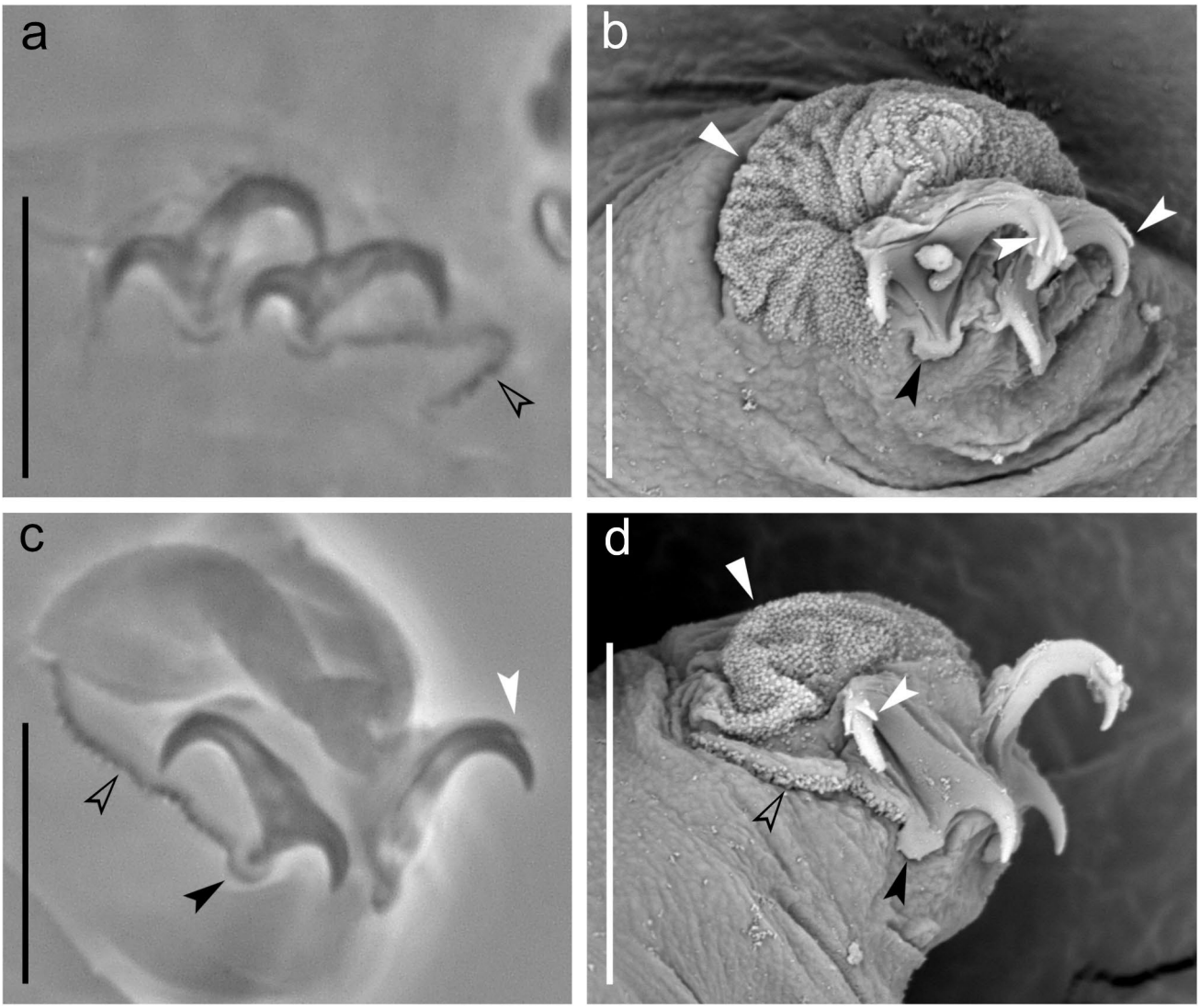


Fig. 7 **a**, **b** Claws of *Eremobiotus alicatai* (topotypic population). External view of leg III showing the external claw of *Isohypsibius* type (claw on the left) and internal claw of the modified *Isohypsibius* claw (claw on the right). **c**, **d** Internal view of leg III showing the external claw of *Isohypsibius* type (claw on the right) and internal claw of the modified *Isohypsibius* claw (claw on the left); empty arrowheads indicate a well-developed single, indented cuticular bar. White indented arrowheads indicate short accessory points; white arrowheads indicate the furbelow-like structure; black arrowheads indicate the poorly indented lunula of the internal (**c**, **d**) and external (**b**) claws. (**a**, **c**, PCM; **b**, **d**, SEM). Scale bars: 10 µm

well-developed indentation in *E. alicatai* while they are only slightly indented in *E. ginevrae*.

The species are also differentiated based on certain morphometric traits (Tables 2, 3, 4, and 5; see also remarks paragraph of *E. ginevrae*).

Eremobiotus alicatai differs from *E. ovezovae* by different dimension and shape of claws on all legs (i.e., bigger and more slender in *E. alicatai*); the external claws of legs I–III more closely resemble *Isohypsibius*-type claws, whereas in *E. ovezovae*, all claws more closely resemble *Eremobiotus*-type claws. The species differ also in morphometric characters (*pt* of all claws).

Amended description of *Eremobiotus ginevrae*

Species: *Eremobiotus ginevrae* Lisi et al., 2016 (Figs. 5g, h; 11).

Material examined

Holotype: Slide No.5530 (Pilato & Binda Collection).

Paratypes of *E. ginevrae*: eight specimens; slide nos. 2388–2391, 5531–5535 (Pilato & Binda Collection).

Amended description

Colorless, cuticle smooth; eye spots present; peribuccal structures look like papular lamellae with PCM (Fig. 11b), but actual morphology needs to be investigated through

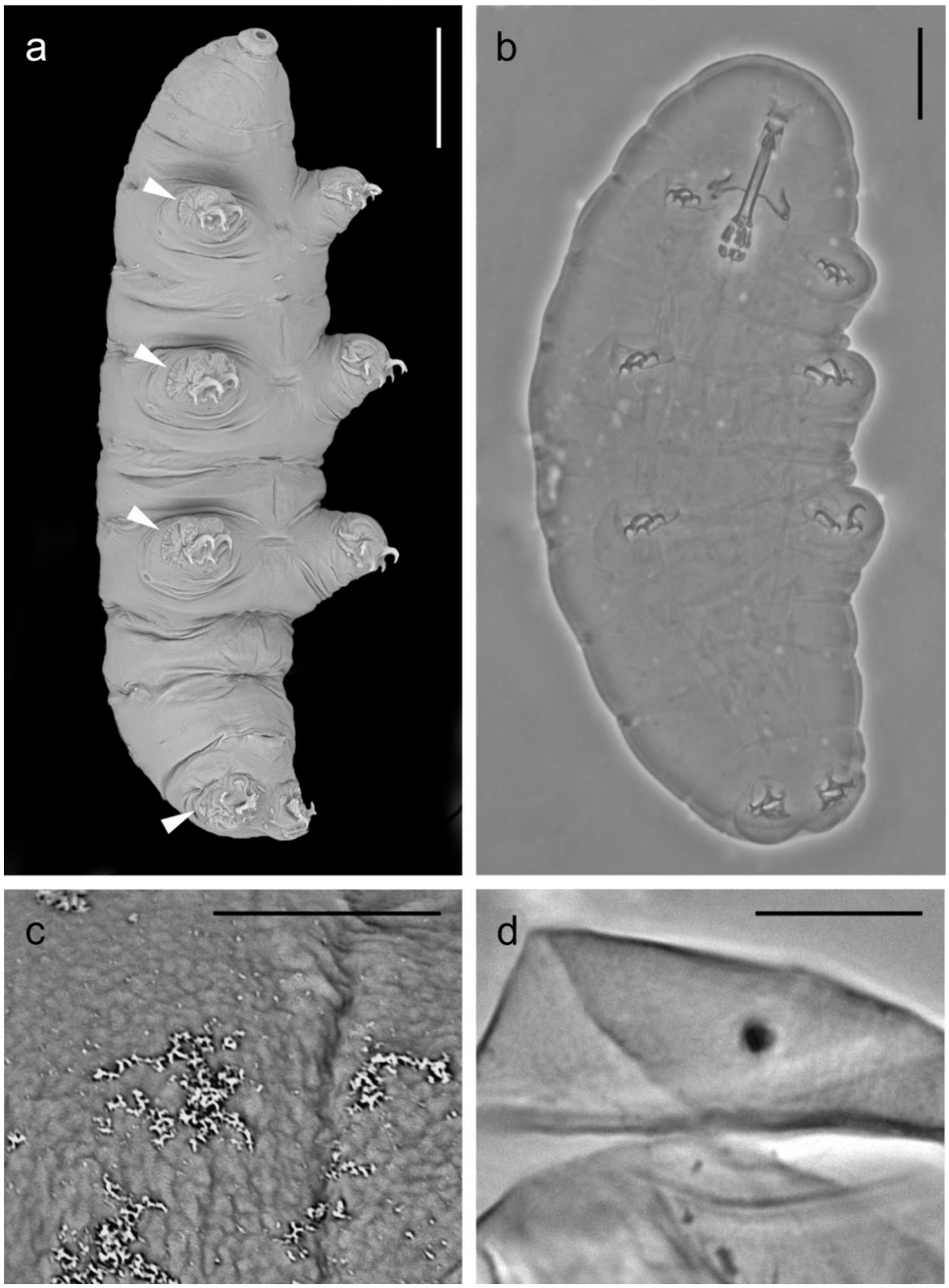


Fig. 8 Habitus and cuticle details of *Eremobiotus alicatai* (**a**, **c**, specimen from topotypic population, Stub DFG2-20; b, d, specimens from topotypic population). **a** Habitus (SEM); white arrowheads indicate well-visible furbelow-like structures on legs. **b** Habitus (PCM, Slide No. 6046). **c** Wrinkled cuticle (SEM, stub no. 16). **d** Wrinkled cuticle (PCM, Slide No. 6025). Scale bar **a**, **b** 25 µm; **c**, **d**: 10 µm

SEM analysis to verify the presence of a continuous buccal ring (as in *E. alicatai*); bucco-pharyngeal apparatus of *Isohypsibius* type (Fig. 11a) with apophyses and two macroplacoids (length sequence 1 > 2) in the pharynx, the first of which showing a central constriction, microplacoid, and septulum absent (Fig. 11c). External claws of the first three pairs of legs of the *Isohypsibius* type; internal claws of the same type but modified as regards the angle between the main and the secondary branches, it being clearly wider (ca. 160–180°) than in the typical *Isohypsibius* type (Fig. 11d). Both anterior and posterior claws of the hind legs of *Eremobiotus*-type (Figs. 5g, h and 11e). Claw accessory points of legs I–III without a visible free portion emerging from the main branches; whereas accessory points of the hind leg claws, in comparison very evident. Lunules on the first three pairs of legs smooth under PCM (Fig. 11d); lunules of the IV pairs of legs slightly indented (Fig. 11e). A furbelow structure, not always well visible under PCM, present on legs I–III (Fig. 11d). The furbelow structures on legs I–III seem to show a granulation under PCM, but a verification with SEM is needed. A long, single cuticular bar with indented margin, present at the base of each internal claw on the first three pairs of legs (Fig. 11d). Eggs smooth laid in the exuviae.

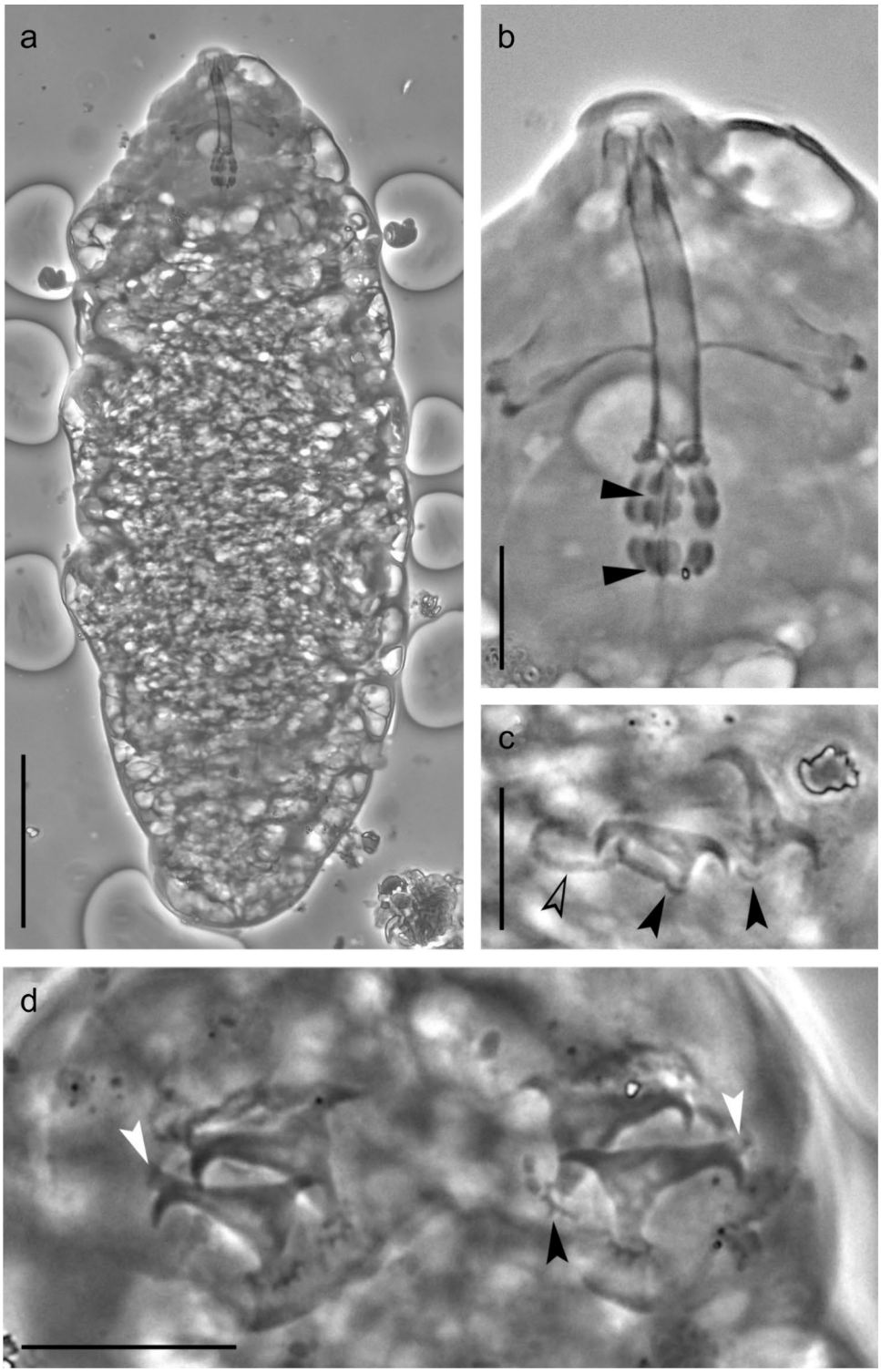


Fig. 9 Holotype of *E. alicatai* (PCM, slide no. 960). **a** Habitus. **b** Bucco-pharyngeal apparatus of *Isohypsibius*-type (flat arrowheads = constrictions of the macroplacoids). **c** Claws of the third pair of legs with a well-visible cuticular bar (empty arrowhead). **d** Claws of the fourth pair of legs with well-developed indented lunules (black indented arrowhead) and accessory points (white indented arrowhead). Scale bar: **a** 50 µm; **b–d** 10 µm

Remarks

The distribution of *E. ginevrae*, as analyzed based on the limited available reports to date (which only refers to observation through PCM), appears to partially overlap with that of *E. alicatai*. The species does not seem to exhibit clear morphological differentiation from *E. alicatai*, with the observed distinctions found in the slightly different indentation of the lunulae of the IV pair of legs (Fig. 5) and of the cuticular bars on the first three leg pairs, and morphometric traits of the buccal apparatus and claws. Notably, the primary branch of the claws in *E. ginevrae* appears slenderer than that of *E. alicatai* (see *E. alicatai*, Fig. 7a, c vs *E.*

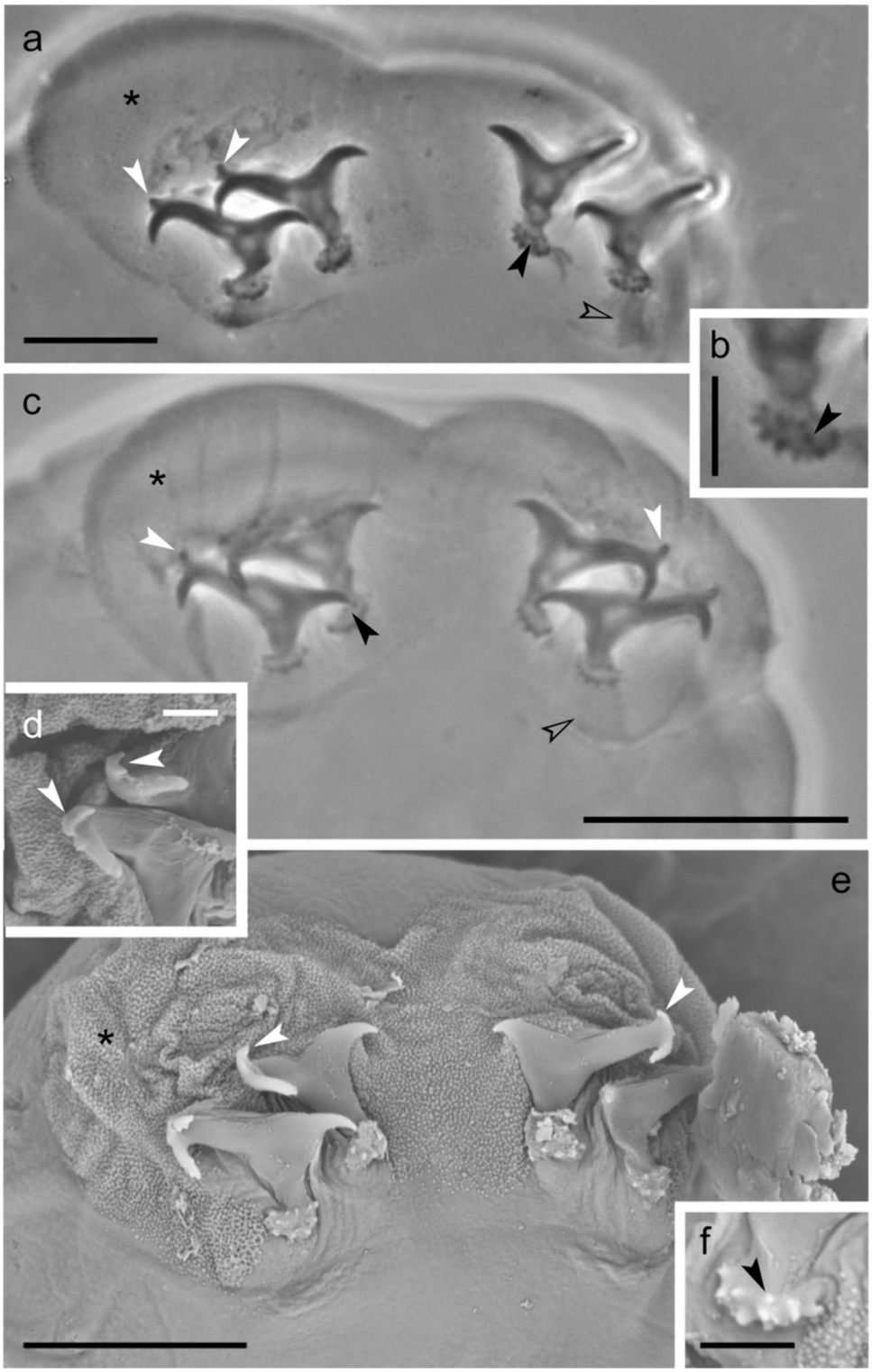


Fig. 10 *Eremobiotus alicatai* IV pair of claws. **a**, **b** Specimen from Orbetello, Fonteblanda (PCM, slide 6065); **c**–**f** specimen from topotypic population (PCM, slide 6046; SEM, Stub DFG2-19). **b** Detail of an indented lunula. **d** Detail of accessory points (SEM). **f** Detail of an indented lunula (SEM). White indented arrowheads indicate accessory points; black indented arrowheads indicate supernumerary teeth of the lunula; asterisk indicates granulation around the claws; empty arrowheads indicate the cuticular thickening at at the base of the lunula. Scale bars **a**, **c**, **e** 10 µm; **b**, **d**, **f** 2 µm

ginevrae and 11d). One of the most relevant distinguishing characters between the two species was the absence of dentate lunules in legs I–III in *E. ginevrae*, but this trait is noted to be not always well observable in *E. alicatai*. The two species also seem to differ in some morphometric characters, even if a partial overlapping is showed: stylets support insertion points *68.7–72.2* in *E. alicatai* vs *71.3–75.5* in *E. ginevrae*, buccal tube external width *11.0–15.2* in *E. alicatai* vs *14.7–19.5* in *E. ginevrae*, external primary branches (claws I) *18.6–22.7* in *E. alicatai* vs *23.7–29.2* in *E. ginevrae*, external primary branches (claws II) *20.8–25.7* in *E. alicatai* vs *25.7–29.6* in *E. ginevrae*, internal primary

Table 2 Measurements (in µm) and *pt* values of selected morphological structures of individuals of *Eremobiotus alicatai* (topotypic population, sample UNICT-DFG2) mounted in Polivinil-Lactophenol medium (*N*—number of specimens/structures measured; *RANGE* refers to the smallest and the largest structure among all measured specimens; *SD*—standard deviation, *pt*—ratio of the length of a given structure to the length of the buccal tube expressed as a percentage). *pt* values are in italic. The claw I–III were measured according to Beasley et al. (2008), the claws IV (*Eremobiotus*-type) were measured according to the newly proposed method (See section "Eremobiotus claw definition and measurement"). Raw measurements are provided in SM.03

Character	*N*	Range		Mean		SD	
		µm	*pt*	µm	*pt*	µm	*pt*
Body length	16	93–378	–	254		65	
Buccal tube length	16	17.6–28.7	–	25.3	–	2.6	–
Stylet support insertion point	16	12.1–20.4	*68.7–72.2*	17.9	*70.7*	1.9	*1.2*
Buccal tube external width	16	2.0–4.1	*11.0–15.2*	3.4	*13.4*	0.6	*1.3*
Buccal tube internal width	16	1.1–3.1	*6.1–11.4*	2.4	*9.4*	0.5	*1.5*
Placoid lengths							
Macroplacoid 1	16	3.0–6.0	*16.2–21.0*	4.7	*18.5*	0.8	*1.5*
Macroplacoid 2	16	2.0–3.9	*10.1–13.8*	3.1	*12.2*	0.5	*1.0*
Macroplacoid row	16	5.3–10.7	*30.2–38.1*	8.7	*34.1*	1.3	*2.1*
Claw I heights							
External base	13	2.5–3.4	*10.1–13.6*	3.1	*12.2*	0.3	*1.1*
External primary branch	12	3.5–6.4	*18.6–22.7*	5.1	*20.8*	0.8	*1.5*
External secondary branch	13	2.4–3.8	*13.2–15.8*	3.5	*14.1*	0.4	*0.7*
External base/primary branch (cct)	11	46.8–70.4	–	58.1	–	8.4	–
Internal base	15	1.8–3.9	*10.0–13.7*	3.0	*11.8*	0.6	*1.2*
Internal primary branch	15	3.6–7.5	*18.6–26.4*	5.2	*20.7*	0.8	*1.9*
Internal secondary branch	13	2.3–3.9	*11.2–13.9*	3.3	*12.8*	0.4	*0.8*
Internal base/primary branch (cct)	15	49.2–65.7	–	57.3	–	5.2	–
Claw II heights							
External base	12	2.7–4.2	*10.6–15.0*	3.3	*12.6*	0.4	*1.3*
External primary branch	13	3.7–7.2	*20.8–25.7*	5.8	*23.3*	0.9	*1.5*
External secondary branch	15	2.5–5.6	*13.0–19.8*	4.0	*15.7*	0.6	*1.6*
External base/primary branch (cct)	10	45.7–59.4	–	52.3	–	4.4	–
Internal base	15	1.9–4.0	*10.7–14.3*	3.1	*12.3*	0.5	*1.2*
Internal primary branch	15	3.6–7.2	*20.3–25.6*	5.9	*23.3*	0.9	*1.5*
Internal secondary branch	14	2.1–4.4	*11.8–15.9*	3.6	*14.2*	0.6	*1.2*
Internal base/primary branch (cct)	15	44.9–60.2	–	52.7	–	4.6	–
Claw III heights							
External base	12	2.9–4.0	*11.2–14.4*	3.4	*13.0*	0.4	*0.9*
External primary branch	11	5.4–7.0	*22.3–27.4*	6.1	*24.0*	0.5	*1.3*
External secondary branch	14	2.5–4.7	*14.3–16.9*	4.0	*15.9*	0.6	*0.9*
External base/primary branch (cct)	10	45.2–59.8	–	54.3	–	4.8	–
Internal base	15	1.9–3.9	*10.8–14.7*	3.1	*12.4*	0.5	*1.1*
Internal primary branch	15	3.8–7.8	*21.2–27.5*	6.0	*23.6*	0.9	*1.8*
Internal secondary branch	13	3.2–4.7	*13.0–17.1*	3.9	*15.0*	0.5	*1.5*
Internal base/primary branch (cct)	15	44.8–66.0	–	52.8	–	5.2	–
Claw IV heights (Eremobiotus claws)							
Anterior branches distance	12	8.9–11.4	*33.8–40.4*	9.8	*37.6*	0.7	*1.7*
Anterior primary branch	12	6.7–8.9	*27.6–32.6*	7.8	*30.0*	0.7	*1.8*
Anterior secondary branch	10	4.4–6.2	*17.2–24.5*	5.3	*20.4*	0.6	*2.2*
Anterior branches distance/primary branch (cct)	12	111.9–134.6	–	125.7	–	7.8	–
Posterior branches distances	12	8.3–11.0	*34.0–40.6*	9.4	*36.6*	0.8	*2.2*
Posterior primary branch	13	6.6–9.5	*27.3–34.1*	8.1	*31.0*	0.9	*2.3*
Posterior secondary branch	13	4.9–7.0	*20.2–25.6*	5.9	*22.5*	0.6	*1.8*
Posterior branches distance/primary branch (cct)	12	104.2–129.6	–	119.1	–	7.7	–

Table 3 Measurements and *pt* values of selected morphological structures of individuals of *Eremobiotus alicatai* (Orbetello population) mounted in Faure medium (slide nos. 6062–6068. Specimen correspondence shown in SM.04) and Hoyer's mounting medium (slide no. 6069. Specimen correspondence shown in SM.04). *N*—number of specimens/structures measured; *RANGE* refers to the smallest and the largest structure among all measured specimens; *SD*—standard deviation. *pt*—ratio of the length of a given structure to the length of the buccal tube expressed as a percentage). *pt* values are in italic. The claws I–III were measured according to Beasley et al. (2008). the claws IV (*Eremobiotus*-type) were measured according to the newly proposed method (See section "Eremobiotus claw definition and measurement"). Raw measurements are provided in SM.04

Character	*N*	Range		Mean		SD	
		µm	*pt*	µm	*pt*	µm	*pt*
Body length	7	209–335	–	284		41	
Buccal tube length	8	23.1–31.1	–	27.8	–	2.4	–
Stylet support insertion point	8	15.7–22.1	*68.3–71.2*	19.5	*70.0*	1.9	*1.0*
Buccal tube external width	8	2.8–3.9	*11.9–14.6*	3.6	*12.9*	0.4	*0.9*
Buccal tube internal width	8	1.7–2.8	*7.3–9.9*	2.3	*8.4*	0.3	*1.1*
Placoid lengths							
Macroplacoid 1	8	4.8–6.4	*19.1–22.6*	5.8	*20.7*	0.6	*1.2*
Macroplacoid 2	8	2.9–4.9	*11.8–15.7*	3.8	*13.6*	0.6	*1.3*
Macroplacoid row	8	8.1–11.8	*34.2–39.1*	10.1	*36.2*	1.0	*1.6*
Claw I heights							
External base	2	3.7–4.0	*13.6–14.2*	3.9	*13.9*	0.3	*0.4*
External primary branch	3	5.1–6.3	*18.1–23.1*	5.8	*20.8*	0.7	*2.5*
External secondary branch	3	3.8–5.1	*13.4–17.1*	4.4	*15.4*	0.7	*1.9*
External base/primary branch (cct)	2	61.6–63.8	–	62.7	–	1.6	–
Internal base	6	2.6–3.6	*11.0–12.2*	3.2	*11.6*	0.4	*0.5*
Internal primary branch	6	4.3–6.4	*18.5–21.8*	5.6	*20.3*	0.8	*1.5*
Internal secondary branch	6	2.6–4.7	*11.1–16.2*	3.8	*13.8*	0.8	*2.1*
Internal base/primary branch (cct)	6	52.9–62.7	–	57.1	–	3.7	–
Claw II heights							
External base	4	3.7–4.0	*13.1–14.5*	3.8	*13.7*	0.1	*0.7*
External primary branch	2	7.7–8.3	*24.8–27.8*	8.0	*26.3*	0.4	*2.1*
External secondary branch	4	4.1–5.3	*14.6–17.9*	4.7	*16.9*	0.5	*1.6*
External base/primary branch (cct)	1	47.6–47.6	–	47.6	–	?	–
Internal base	6	2.6–4.3	*11.4–14.4*	3.7	*13.2*	0.6	*1.1*
Internal primary branch	3	5.2–7.3	*22.6–24.7*	6.4	*23.6*	1.1	*1.0*
Internal secondary branch	7	3.0–5.6	*12.8–18.8*	4.3	*15.5*	0.8	*2.2*
Internal base/primary branch (cct)	3	50.6–54.1	–	52.0	–	1.8	–
Claw III heights							
External base	4	3.4–4.4	*12.3–14.3*	3.9	*13.6*	0.4	*0.9*
External primary branch	1	6.4–6.4	*22.9–22.9*	6.4	*22.9*	?	*?*
External secondary branch	4	4.5–5.5	*15.9–20.3*	5.0	*17.8*	0.5	*1.8*
External base/primary branch (cct)	0	?	–	?	–	?	–
Internal base	4	2.6–4.0	*11.4–14.3*	3.4	*12.7*	0.6	*1.3*
Internal primary branch	6	5.7–8.4	*20.8–27.0*	6.8	*24.5*	1.1	*2.1*
Internal secondary branch	3	4.1–4.8	*14.5–17.6*	4.5	*16.1*	0.3	*1.5*
Internal base/primary branch (cct)	4	46.4–56.9	–	53.6	–	4.9	–
Claw IV heights (Eremobiotus claws)							
Anterior branches distance	6	9.7–12.0	*33.5–40.9*	10.8	*37.3*	0.9	*3.5*
Anterior primary branch	5	7.8–8.9	*28.1–29.3*	8.3	*28.7*	0.4	*0.5*
Anterior secondary branch	5	5.6–6.6	*19.7–22.2*	6.0	*20.7*	0.4	*1.1*
Anterior branches distance/primary branch (cct)	5	118.8–143.3	–	132.8	–	10.5	–
Posterior branches distance	5	8.5–12.1	*30.4–40.9*	10.3	*35.4*	1.3	*4.0*
Posterior primary branch	4	6.6–9.8	*23.6–33.1*	8.4	*28.5*	1.4	*3.9*
Posterior secondary branch	6	5.9–10.3	*21.1–36.9*	7.5	*25.9*	1.5	*5.6*
Posterior branches distance/primary branch (cct)	4	117.4–128.5	–	122.5	–	4.7	–

Table 4 Measurements and *pt* values of selected morphological structures of individuals of *Eremobiotus alicatai* (type series) mounted in Polivinil-Lactophenol medium (*N*—number of specimens/structures measured; *RANGE* refers to the smallest and the largest structure among all measured specimens; *SD*—standard deviation, *pt*—ratio of the length of a given structure to the length of the buccal tube expressed as a percentage). *pt* values are in italic. The claws I–III were measured according to Beasley et al. (2008), the claws IV (*Eremobiotus*-type) were measured according to the newly proposed method (See section "Eremobiotus claw definition and measurement"). Raw measurements are provided in SM.05

Character	*N*	Range		Mean		SD		Holotype	
		µm	*pt*	µm	*pt*	µm	pt	µm	pt
Body length	2	248–258	–	253		7		258	
Buccal tube length	3	26.1–27.7	–	26.8	–	0.9	–	26.5	–
Stylet support insertion point	3	18.6–19.6	*70.2–72.1*	19.0	*71.0*	0.5	*1.0*	18.6	*70.2*
Buccal tube external width	3	3.8–4.0	*14.2–14.6*	3.9	*14.5*	0.1	*0.2*	3.9	*14.6*
Buccal tube internal width	3	3.0–3.1	*11.1–11.6*	3.0	*11.4*	0.0	*0.3*	3.1	*11.5*
Placoid lengths									
Macroplacoid 1	3	5.1–5.6	*18.4–21.7*	5.3	*19.7*	0.3	*1.7*	5.1	*19.2*
Macroplacoid 2	3	3.1–3.6	*11.8–14.0*	3.3	*12.5*	0.3	*1.3*	3.1	*11.8*
Macroplacoid row	3	9.1–10.3	*33.3–39.5*	9.5	*35.7*	0.7	*3.4*	9.1	*34.2*
Claw I heights									
External base	2	3.7–3.9	*14.3–14.7*	3.8	*14.5*	0.1	*0.3*	3.9	*14.7*
External primary branch	3	5.3–5.6	*19.0–21.6*	5.4	*20.3*	0.2	*1.3*	5.4	*20.3*
External secondary branch	3	3.8–3.9	*14.0–14.8*	3.9	*14.4*	0.1	*0.4*	3.9	*14.8*
External base/primary branch (cct)	2	66.2–72.0	–	69.1	–	4.1	–	72.0	–
Internal base	3	3.2–3.2	*11.4–12.1*	3.2	*11.9*	0.0	*0.4*	3.2	*12.1*
Internal primary branch	3	5.0–5.8	*18.1–21.7*	5.3	*19.7*	0.4	*1.8*	5.8	*21.7*
Internal secondary branch	3	3.2–3.9	*12.2–15.0*	3.5	*13.1*	0.3	*1.6*	3.2	*12.2*
Internal base/primary branch (cct)	3	55.6–63.3	–	60.6	–	4.3	–	55.6	–
Claw II heights									
External base	3	3.6–4.1	*12.9–15.3*	3.8	*14.2*	0.3	*1.2*	4.1	*15.3*
External primary branch	3	5.6–7.0	*21.6–26.5*	6.2	*23.3*	0.7	*2.8*	7.0	*26.5*
External secondary branch	3	4.1–4.5	*15.3–16.8*	4.3	*16.0*	0.2	*0.8*	4.5	*16.8*
External base/primary branch (cct)	3	57.9–65.6	–	61.1	–	4.0	–	57.9	–
Internal base	3	3.1–3.4	*11.2–12.8*	3.3	*12.2*	0.2	*0.9*	3.4	*12.8*
Internal primary branch	3	5.6–6.4	*20.2–24.3*	6.1	*22.6*	0.4	*2.2*	6.4	*24.3*
Internal secondary branch	3	4.0–4.0	*14.4–15.4*	4.0	*14.9*	0.0	*0.5*	4.0	*14.9*
Internal base/primary branch (cct)	3	52.8–55.4	–	54.2	–	1.3	–	52.8	–
Claw III heights									
External base	2	3.7–4.0	*14.0–14.9*	3.8	*14.4*	0.2	*0.6*	4.0	*14.9*
External primary branch	2	6.4–6.8	*24.5–25.5*	6.6	*25.0*	0.3	*0.7*	6.8	*25.5*
External secondary branch	2	4.3–4.4	*16.4–16.5*	4.3	*16.4*	0.0	*0.1*	4.4	*16.4*
External base/primary branch (cct)	2	3.4–57.2	–	30.3	–	38.1	–	3.4	–
Internal base	2	3.2–3.4	*12.2–12.7*	3.3	*12.4*	0.1	*0.3*	3.4	*12.7*
Internal primary branch	3	5.9–6.5	*22.8–24.6*	6.3	*23.6*	0.3	*0.9*	6.5	*24.6*
Internal secondary branch	3	3.9–4.6	*14.9–16.5*	4.2	*15.5*	0.4	*0.9*	4.0	*15.1*
Internal base/primary branch (cct)	2	51.5–53.5	–	52.5	–	1.4	–	51.5	–
Claw IV heights (Eremobiotus claws)									
Anterior branches distance	3	9.5–9.9	*34.2–38.1*	9.8	*36.5*	0.2	*2.0*	9.9	*37.2*
Anterior primary branch	3	7.5–7.9	*27.4–30.4*	7.7	*28.7*	0.2	*1.6*	7.5	*28.2*
Anterior secondary branch	3	5.6–6.0	*21.3–22.8*	5.9	*21.9*	0.2	*0.8*	6.0	*22.8*
Anterior branches distance/primary branch (cct)	3	124.6–131.6	–	127.1	–	3.9	–	131.6	–
Posterior branches distance	3	9.4–9.7	*34.1–36.6*	9.6	*35.7*	0.1	*1.4*	9.7	*36.6*
Posterior primary branch	3	7.8–8.6	*28.2–32.3*	8.1	*30.3*	0.4	*2.0*	8.6	*32.3*
Posterior secondary branch	3	6.3–6.7	*23.1–25.3*	6.5	*24.2*	0.2	*1.1*	6.7	*25.3*
Posterior branches distance/primary branch (cct)	2	113.4–120.5	–	117.0	–	5.0	–	113.4	–

Table 5 Measurements and *pt* values of selected morphological structures of individuals of *Eremobiotus ginevrae* (type series) mounted in Polivinil-Lactophenol medium (*N*—number of specimens/structures measured; *RANGE* refers to the smallest and the largest structure among all measured specimens; *SD*—standard deviation. *pt*—ratio of the length of a given structure to the length of the buccal tube expressed as a percentage). *pt* values are in italic. The claws I–III were measured according to Beasley et al. (2008). the claws IV (*Eremobiotus*-type) were measured according to the newly proposed method (See section "Eremobiotus claw definition and measurement"). Raw measurements are provided in SM.06

Character	*N*	Range		Mean		SD		Holotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	7	181–357	–	253		64		324	
Buccal tube length	7	24.7–32.5	–	27.4	–	3.3	–	32.5	–
Stylet support insertion point	7	18.2–23.7	*71.3–75.5*	20.0	*73.0*	2.3	*1.4*	23.7	*72.7*
Buccal tube external width	6	3.7–6.3	*14.7–19.5*	4.3	*15.8*	1.0	*1.8*	6.3	*19.5*
Buccal tube internal width	6	2.7–5.0	*10.6–15.4*	3.3	*12.2*	0.9	*1.7*	5.0	*15.4*
Placoid lengths									
Macroplacoid 1	7	4.3–7.3	*17.3–22.4*	5.5	*19.9*	1.1	*2.0*	7.3	*22.4*
Macroplacoid 2	7	2.6–5.1	*10.7–15.7*	3.5	*12.7*	0.9	*1.6*	5.1	*15.7*
Macroplacoid row	7	8.0–13.5	*32.2–41.6*	9.9	*35.9*	2.2	*3.7*	13.5	*41.6*
Claw I heights									
External base	7	3.0–5.3	*12.2–16.5*	4.0	*14.5*	0.8	*1.4*	4.9	*14.9*
External primary branch	6	6.3–9.5	*23.7–29.2*	7.4	*26.7*	1.5	*2.2*	9.5	*29.2*
External secondary branch	6	3.6–6.6	*13.6–20.2*	4.8	*17.0*	1.1	*2.2*	6.6	*20.2*
External base/primary branch (cct)	6	45.6–62.9	–	53.7	–	6.0	–	51.2	–
Internal base	6	3.3–5.3	*12.8–16.2*	4.0	*14.2*	0.8	*1.2*	5.3	*16.2*
Internal primary branch	6	5.7–9.7	*21.6–29.8*	6.9	*24.8*	1.6	*2.8*	9.7	*29.8*
Internal secondary branch	6	3.8–5.3	*14.3–17.0*	4.4	*16.0*	0.7	*1.1*	5.3	*16.2*
Internal base/primary branch (cct)	6	52.9–60.3	–	57.4	–	3.1	–	54.3	–
Claw II heights									
External base	6	3.0–4.9	*12.2–15.4*	4.0	*14.4*	0.8	*1.3*	4.8	*14.8*
External primary branch	3	6.6–9.6	*25.7–29.6*	7.6	*27.4*	1.7	*2.0*	9.6	*29.6*
External secondary branch	5	3.8–6.3	*15.5–19.4*	5.0	*17.5*	1.1	*1.5*	6.3	*19.4*
External base/primary branch (cct)	3	45.6–52.1	–	49.2	–	3.3	–	50.0	–
Internal base	5	3.3–5.3	*13.2–16.3*	4.2	*14.7*	0.8	*1.1*	5.3	*16.3*
Internal primary branch	6	6.2–9.7	*25.0–29.8*	7.4	*26.8*	1.3	*1.9*	9.7	*29.8*
Internal secondary branch	6	3.8–5.3	*15.2–18.0*	4.6	*16.6*	0.6	*1.0*	5.3	*16.3*
Internal base/primary branch (cct)	5	52.9–55.7	–	54.3	–	1.1	–	54.6	–
Claw III heights									
External base	5	3.0–5.2	*12.1–16.1*	4.1	*14.3*	0.9	*1.6*	5.2	*16.1*
External primary branch	6	6.7–9.8	*26.8–30.2*	7.8	*28.1*	1.3	*1.2*	9.8	*30.2*
External secondary branch	5	3.9–6.6	*15.8–20.2*	5.1	*18.1*	1.1	*1.7*	6.6	*20.2*
External base/primary branch (cct)	5	43.6–54.1	–	50.5	–	4.2	–	53.2	–
Internal base	6	3.4–5.2	*13.5–16.0*	4.0	*14.4*	0.8	*0.9*	5.2	*16.0*
Internal primary branch	6	6.4–9.8	*24.7–30.0*	7.4	*26.7*	1.3	*1.9*	9.8	*30.0*
Internal secondary branch	6	3.6–6.3	*14.6–19.4*	4.6	*16.6*	1.1	*1.8*	6.3	*19.4*
Internal base/primary branch (cct)	6	51.7–59.6	–	54.0	–	2.8	–	53.2	–
Claw IV heights (Eremobiotus claws)									
Anterior branches distance	6	8.8–14.6	*35.6–44.9*	10.9	*39.0*	2.3	*3.5*	14.6	*44.9*
Anterior primary branch	7	6.9–11.7	*27.8–35.9*	8.4	*30.5*	1.7	*2.8*	11.7	*35.9*
Anterior secondary branch	6	4.4–7.9	*17.6–24.3*	5.6	*20.1*	1.4	*2.6*	7.9	*24.3*
Anterior branches distance/primary branch (cct)	6	122.0–132.0	–	126.1	–	3.5	–	125.0	–
Posterior branches distance	5	8.6–14.4	*34.8–44.2*	10.8	*37.9*	2.5	*3.9*	14.4	*44.2*
Posterior primary branch	5	6.4–12.3	*25.5–37.8*	8.7	*30.5*	2.4	*4.8*	12.3	*37.8*
Posterior secondary branch	7	4.7–8.4	*19.0–25.9*	5.8	*21.1*	1.4	*2.3*	8.4	*25.9*
Posterior branches distance/primary branch (cct)	5	115.2–136.9	–	125.1	–	9.0	–	117.0	–

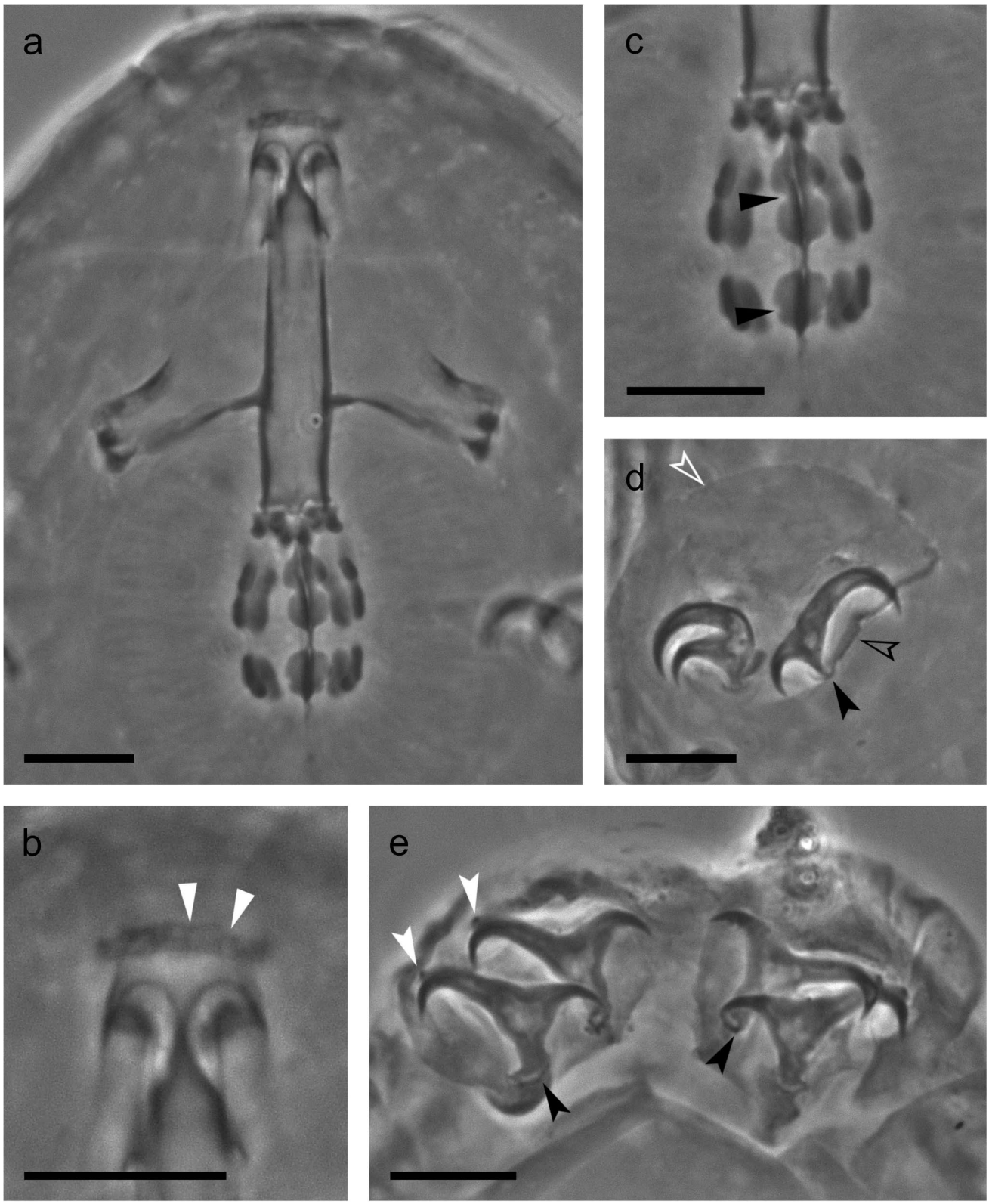


Fig. 11 Holotype of *E. ginevrae* (PCM). **a** Bucco-pharyngeal apparatus of *Isohypsibius*-type. **b** Buccal opening with apparent papular-lamellae. **c** Detail of the placoids (arrows indicate respectively the medial constriction of the first macroplacoid and the subterminal constriction of the second macroplacoid). **d** Claws of the third pair of legs with a well-visible cuticular bar (empty indented arrowhead) and apparently smooth lunula of the internal claw (black indented arrowhead). **e** Claws of the fourth pair of legs with indented lunules (black indented arrowheads) and accessory points (white indented arrowheads)

branches (claws II) *20.3–25.6* in *E. alicatai* vs *25.0–29.8* in *E. ginevrae*, internal secondary branches (claws II) *11.8–15.9* in *E. alicatai* vs *15.2–18.0* in *E. ginevrae*, external primary branches (claws III) *22.3–27.4* in *E. alicatai* vs *26.8–30.2* in *E. ginevrae*.

Furthermore, the limited number of specimens from the type series, coupled with their less-than-optimal preservation conditions, prevents a good analysis of some important morphological characters (i.e., peribuccal structures, AISM, indentation of lunulae). To gain a deeper understanding of *E. ginevrae*, it is imperative to discover specimens in their *locus typicus* and conduct an integrated study. The two Sicilian species could be the same taxonomical entity, and a comprehensive and modern approach is essential to elucidate their relationship.

Remarks on *Eremobiotus ovezovae*

Species: *Eremobiotus ovezovae* Biserov, 1992

Material examined

A single paratype of *E. ovezovae* (slide No. 3358), donated by Biserov, was examined and measured (SM.07).

Remarks

The species, only reported from Turkmenistan, exhibits a distinctive claw morphology, markedly different from the other known *Eremobiotus* species. The examination of the paratype with PCM revealed a likely similar situation to the other two species of *Eremobiotus* concerning peribuccal structures and claws. A circular sensory field is visible with PCM; peribuccal structures look like papular lamellae with PCM, but it is essential to check the morphology through SEM analysis to verify the presence of a continuous buccal ring (as in *E. alicatai*). Internal claws of legs I–III of modified *Isohypsibius*-type (with an angle between branches of ca. 160–180°) do not always exhibit a clear 180° angle between branches; instead, they often have a narrower angle.

The redescription of *E. ovezovae* may lead to different scenarios on the peribuccal structures, including:

1. Confirmation of the hypothesis that *E. ovezovae* possesses a continuous buccal ring and 6 peribuccal lobes with SEM, alongside maintaining all three species within the *Eremobiotus* genus, if supported by molecular data
2. Confirmation of the hypothesis that *E. ovezovae* has a continuous buccal ring and 6 papular peribuccal lobes with SEM (similarly to the *Calohypsibius* peribuccal structures arrangement), but leading to the establishment of a new genus for *E. alicatai* and *E. ginevrae*, if supported by molecular data
3. Confirmation of Biserov's description, i.e., the presence of papular lamellae and 6 peribuccal lobes, which might prompt the erection of a new genus for *E. alicatai* and *E. ginevrae*, if also supported by molecular data

Depending on further analysis on this interesting species, the scenario concerning *Eremobiotus* composition could change.

Molecular analysis

The phylogenetic tree based on Bayesian inference and maximum likelihood analyses was computed on 3566 bp of the 18S and 28S rRNA genes of specimens belonging to the superfamily Isohypsibioidea (Fig. 12). It shows that all newly analyzed specimens are encompassed in a single highly supported lineage. The latter shows *Ursulinius* specimens placed in a basal position, whereas *Eremobiotus* specimens are closely related to *Dastychius improvisus*. All *Eremobiotus* specimens cluster together in a highly supported group, thus confirming the validity of the genus (Fig. 12). Furthermore, the analysis of specimens of the type locality of the genus confirms that previously analyzed specimens from Orbetello (Bertolani et al., 2014) and Hungary (Gąsiorek et al., 2019a) pertain to the *Eremobiotus* genus, whereas other specimens from USA previously analyzed (Guil & Giribet, 2012) were incorrectly assigned to *Eremobiotus* thus needing a further revision, as already pointed out by Mioduchowska et al. (2021). For this reason, these specimens were not included in the present analysis.

Species delimitation analyses were performed both on the COI and the ITS-2 markers, revealing different degrees of variability: for the former gene, a high intraspecific variability was scored (*p*-distances between 0 and 8.8%; SM.08), whereas for the ITS2 marker, *p*-distances among all *Eremobiotus* specimens were decidedly lower (between 0 and 0.6%; SM.09). Therefore, the COI dataset is the most complete and informative for species delimitation investigation, and a phylogenetic tree computed with the maximum likelihood method of the *E. alicatai* specimens was utilized for the PTP analysis, showing three putative species clusters: the first including one specimen from Orbetello, the second comprising two specimens from Orbetello, and the third including three specimens from Gela and one from Orbetello. This subdivision is validated by the haplotype network, but not by ASAP analysis (Fig. 13). Given the absence of evident morphological differences among specimens characterizing the two populations and the fact that all species delimitation analyses are not in agreement, at the moment, the most parsimonious option is to consider all analyzed specimens as pertaining to the same species with a high intraspecific variability. However, it cannot be ruled out that the species may conceal a species complex, considering that *E. alicatai* has been

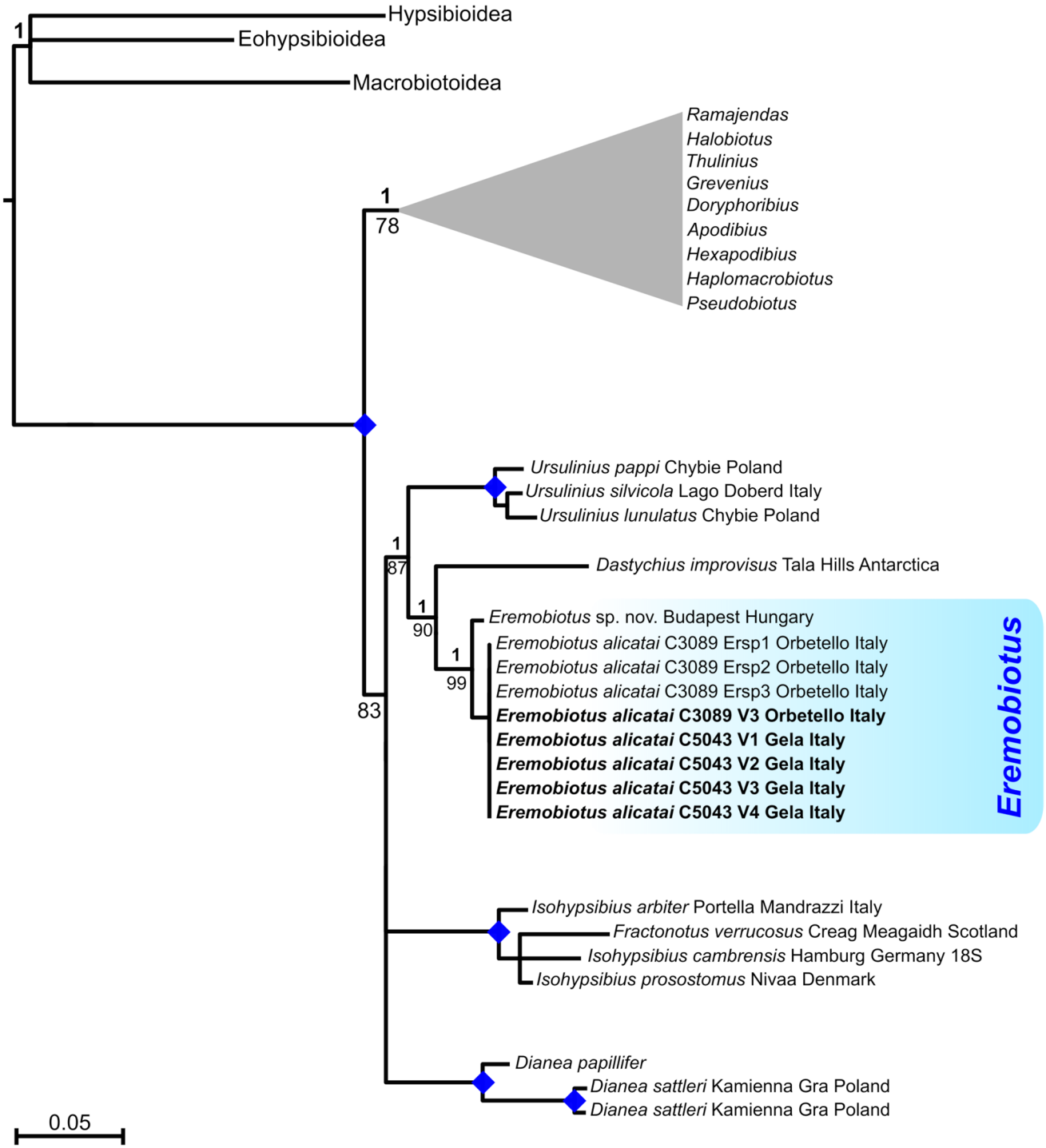


Fig. 12 Bayesian inference and maximum likelihood phylogenetic tree based on 3566 bp of the 18S and 28S rRNA genes in specimens pertaining to the superfamily Isohypsibioidea. Numbers above and below nodes indicate Bayesian inference posterior probability values (pp) and bootstrap support values (bs), respectively. Nodes with maximum support for both analyses (pp = 1.0; bs = 100) are marked with a blue diamond. All nodes with pp < 0.98 and bs < 75% are collapsed. Newly analyzed specimens are shown in bold

found in several places in the Palearctic region, and previous reports lack an integrative approach. Therefore, new investigations on these specimens/populations could help in clarifying this scenario.

Conclusion

The present study introduced new nomenclature terms for eutardigrade buccal opening structures ("papular peribuccal

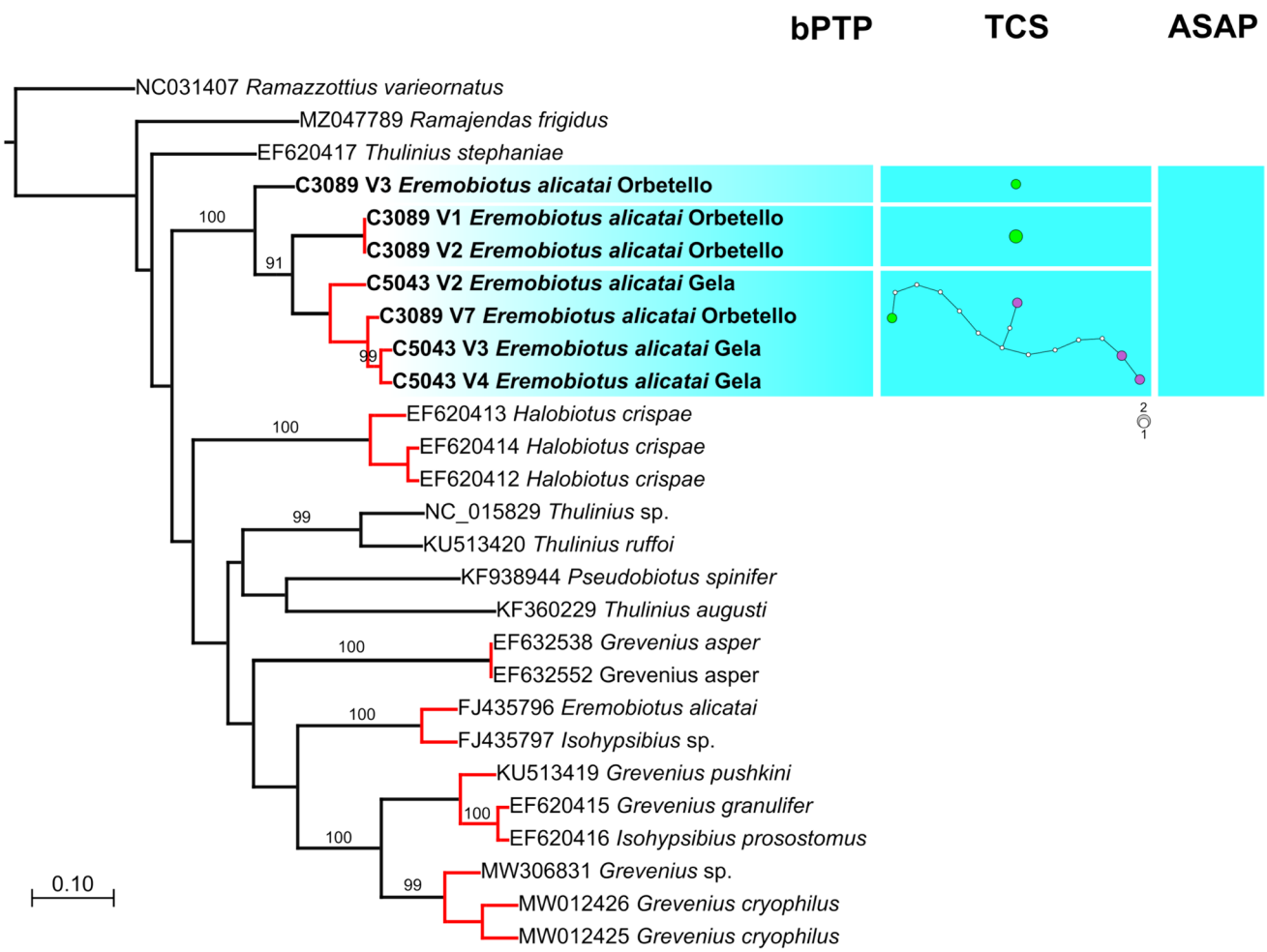


Fig. 13 Species delimitation analyses based on 788 bp of the COI gene of *Eremobiotus alicatai*; (left) tree resulting from the maximum likelihood analysis. Values above branches represent bootstrap values. Results of the Poisson tree process analysis are provided using differently colored branches: putative species are indicated using transitions from black-colored to red-colored branches. The scale bar shows the number of substitutions per nucleotide position; (center) Haplotype network analysis. Green and purple circles denote haplotypes, while circle surface represents haplotype frequency. White circles show putative/missing haplotypes. Networks falling below the value of the 95% connection limit are disconnected; (right) ASAP analysis showing different specimens falling into groups indicated by rectangles and subdivided by a threshold of 10.51% as supported by the lowest ASAP score (2.00). Newly analyzed specimens are shown in bold

lobes" and "papular lamellae"), in the attempt to clarify the sometimes-confusing definition of "peribuccal papulae" from previous literature, discussing and defining them based on their position and probable homology.

Thanks to a morphological analysis of types of the three species of *Eremobiotus* (*E. alicatai*, *E. ginevrae*, *E. ovezovae*), it was possible to rectify and update the diagnostic characters for the genus, with a clear definition of the "*Eremobiotus*-type" claws, and a novel measurement method for facilitating the morphometric study of the claws of *Eremobiotus* species. All this also allowed to redescribe *E. alicatai* (integrating with new PCM, SEM, and genetic material) and to amend the description of *E. ginevrae*. Actual morphology of the buccal ring of these two species, requiring new fresh material for SEM analysis, is still to be confirmed in order to complete the species and genus description/definition.

The molecular analysis of four genes (18S, 28S, COI, and ITS2) contributed to correct the phylogenetic position of the genus and taxonomy of *E. alicatai* with new information on its distribution, but questions remain pending regarding potential cryptic species, and the other species of the genus are still in need to be investigated molecularly.

The present results further highlight the integrative approach as the best one to allow a more complete insight on tardigrade biodiversity.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s13127-024-00657-8.

Acknowledgements We are grateful to Prof. Puglisi for moss identification and Dr. Siracusa for sputter coating service.

Author contribution DC, FF, RG, MC, OL: conceptualization, methodology. DC: morphological and morphometric data collection. FF: molecular data collection. DC: analysis of the morphological and morphometric data. FF, MC: analysis of the molecular data. DC: writing (original draft) preparation. DC, RG, MC, FF, OL: writing (review and editing). All authors contributed to the final manuscript and approved its submission.

Funding Open access funding provided by Università degli Studi di Modena e Reggio Emilia within the CRUI-CARE Agreement. This work was partially funded by the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 – Call for tender No. 3138 of 16 December 2021, rectified by Decree n. 3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU. Project Code [CN_00000033], Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP E93C22001090001, Project title "National Biodiversity Future Center – NBFC".

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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