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A new, isolated population of *Dichotrachelus meregallii* (Coleoptera: Curculionidae) from the Ortler massif (South Tyrol, Eastern Alps)

Abstract

The discovery of an isolated population of *Dichotrachelus meregallii* in the Eastern Alps, in South Tyrol, has stimulated a study, based on morphological and molecular analyses, to compare the new find with the typical population of the Western Alps. The results indicate that the two populations differ only by very few substitutions in the mitochondrial genomic sequences, that do not warrant taxonomic recognition. The new data suggest that the species was originally diffused all along the Alpine chain, and its present relict distribution was probably determined by the Pleistocene climatic events. The phylogenetic relationships of *D. ulbrichi*, originally referred to the *D. meregallii* group, were also analysed. Based on its mitochondrial genomic sequences, it belongs to the *D. rudeni* group.

Kewyords: Bayesian inference, distribution, molecular taxonomy, relict taxa, South Tyrol fauna

1. Introduction

Dichotrachelus meregallii Osella, 1971 was described based on 3 specimens from two localities at high altitude in the Western Alps (Osella 1971). Since then, this relatively uncommon species has been found in some more localities between the higher Susa Valley and the Gran Paradiso massif (Meregalli, unpublished data).

With great surprise, we were informed that Gerd Müller (Frechen, Germany) casually found a male specimen of a *Dichotrachelus* at 2600 m a.s.l. along the ski run near Schaubachhütte, south of Solda (Sulden), South Tyrol (46°29'22"N 10°35'58"E, 2603 m), that was not the common and rather widespread *D. stierlini* Gredler, 1856, but rather was morphologically and by the shape of the genitalia very similar to *D. meregallii*. This specimen was examined by two of the authors (M.M. and M.K.), who confirmed the provisional identification (Fig. 1a). An initial subsequent survey carried out by M.K. in 2017 failed to discover more specimens. On July 7, 2018 M.K., together with Alexander Szallies (Zürich, Switzerland), collected some more specimens between Schaubachhütte and Madritschhütte and near Madritschhütte (46°29'18"N 10°36'16"E, 2703 m and 46°29'48"N 10°36'48"E, 2819 m). More specimens were found a few days later by Szallies, and again on July 31, 2018, by M.M., including also larvae and pupae, above Düsseldorferhütte, in Zaytal, north-east of Solda (46°32'55.50"N 10°37'34.51"E, 2760 m).

The relationships between the typical form of *Dichotrachelus meregallii* from the Western Alps and the newly discovered population from the Eastern Alps were investigated through a morphological analysis combined with the analysis of the mitochondrial cytochrome oxidase I (mt-CoxI).

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2. Materials and methods

2.1 Specimens collection

The specimens of the eastern population used for the molecular analysis (larvae, pupae and a freshly hatched adult) were sampled during the research expedition carried out on July 31, 2018. The previously collected specimens cited in the introduction were used for the morphological analysis.

Those of the western population were sampled at Rifugio Casa di Caccia del Gran Piano in the Gran Paradiso Park (45°28'18.70"N, 7°17'28.97"E, 2252 m) in July 2015 by M.M., Cristiana Cerrato and Alessandro Girodo.

All specimens were collected on mosses on large boulders (Figures 1b–1d). They were stored in vials with alcohol 96 % immediately after collection and conserved at minus 23°C until they were processed. Specimens from previous collections had been conserved dry on card in entomological drawers.

A larva of *Dichotrachelus ulbrichi* was added to the study, collected at the type locality, the Kärntner Storschitz, near Eisenkappel, in southern Austria (46°25'54"N 14°31'32"E, 1677 m).

2.2 Morphological analysis.

Specimens were examined under a Leica 6SE stereomicroscope. Genitalia were cleared with 10 % KOH and carefully dissected; female genitalia and the male genital sclerite

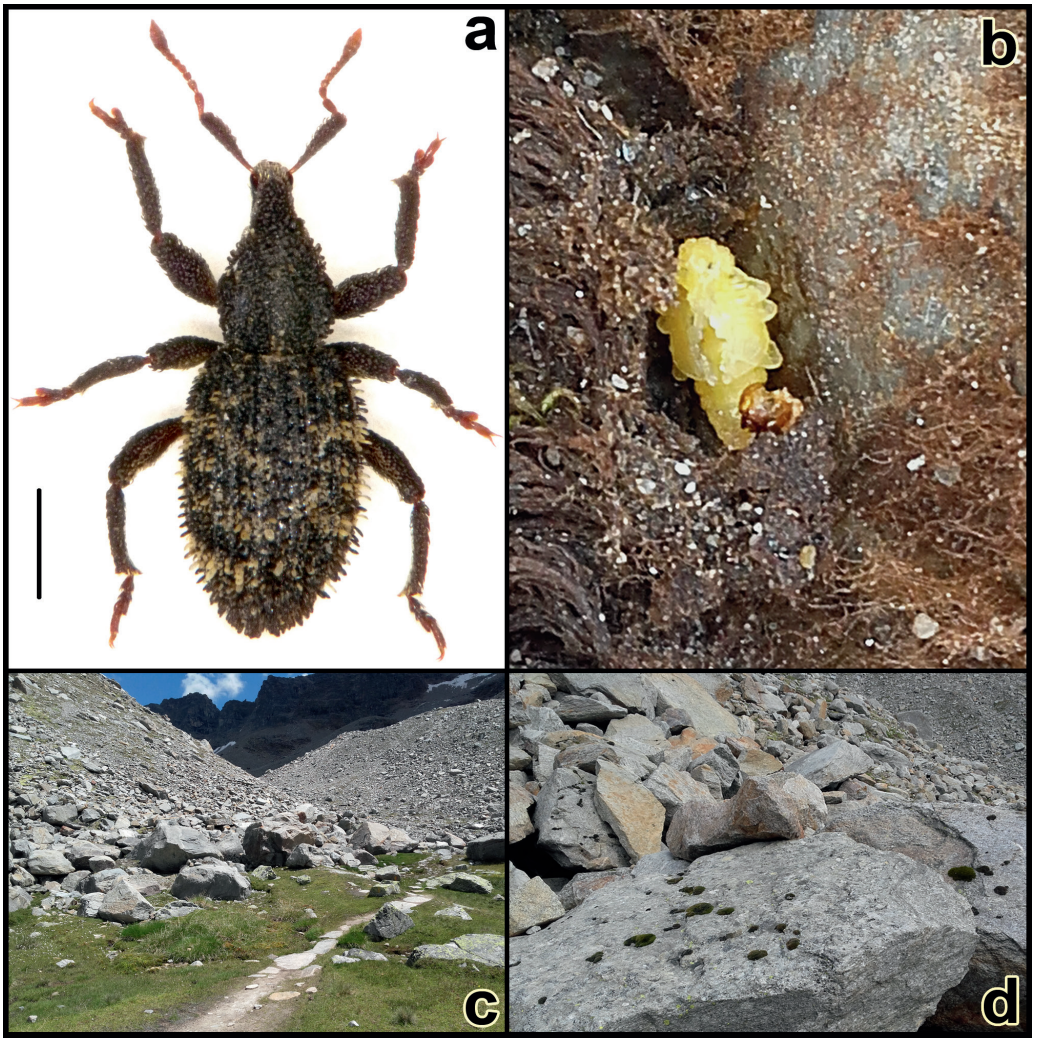

Fig. 1: a. *Dichotrachelus meregallii*, ♂, Solda, Schaubachhütte, leg. Müller; bar: 1mm. **b.** *Dichotrachelus meregallii.* Pupa, Düsseldorferhütte, leg. Meregalli. **c.** Habitat at Düsseldorferhütte. **d.** Large rocks with cushions of mosses, the typical habitat of *D. meregallii.*

were embedded in resin, male genitalia were mounted dry on the same card as the respective specimen. Photographs of the body were taken using a Nikon P 6000 digital camera mounted on the same stereomicroscope, combining image stacks with Zerene Stacker; images were cleaned and enhanced as necessary in Adobe Photoshop CS3.

2.3 Phylogenetic analysis

Total DNA was extracted by placing the sample in 400 µl of 5M guanidine-isothiocyanate, after separating head+pronotum for the adult specimens and the cephalic capsule from the rest of the body for the larvae. DNA extraction was conservative for the adults and destructive for the larvae. Two different regions of the mt-CoxI gene were amplified. One region was amplified with primers based on Hughes & Vogler (2004): fw C1-J-2183 (Jerry), 5'-CAACATTTATTTTGATTTTTTGG-3' and rev L2-N-3014 (Pat), 5'-TCCAATGCACTAATCTGCCATATTA-3'. The other region was amplified with primers based on Folmer et al. (1994) modified as in Astrin & Stüben (2008): fw: LCO1490-JJ, CHACWAAYCATAAAGATATYGG; rev: HCO2198-JJ, AWACTTCVGGRTGVCCAAARAATCA. Amplification of DNA was done as follows: 15 min of initial denaturation (95°C) followed by 10 cycles of 30 sec at 94°C, 45 sec at 60°C to 50°C (lowering the annealing temperature in each cycle 1°C), 2 min at 72°C followed by 30 cycles of 30 sec at 94°C, 45 sec at 50°C, 2 min at 72°C and a final extension cycle of 15 min at 72°C. The reaction products were purified by agarose gel electrophoresis and successive purification from the gel. Sequencing was performed by an external service (Genechron, Roma). Both strands were sequenced. Forward and reverse chromatograms were checked with Chromas (http://technelysium.com.au /wp/chromas/) using default parameters, and ambiguities (occasional double peaks or very few sites with uncertainties due to a low credibility value) were checked and when necessary corrected manually. The mt-CoxI sequences had no indels after alignment, no stop codons were detected and the translation to the amino acid was congruent with the protein sequence known for the other species of the genus. In the here presented framework this was considered as sufficient evidence against the presence of NUMT (nuclear mitochondrial DNA) pseudogenes. The two sequences obtained with the two pairs of primers were analysed independently. Multiple sequence alignment of both strands was performed with MEGA-X (Kumar et al. 2018), after reversing and complementing the reverse strand, with the Muscle alignment option. The sequences were trimmed at the extremes before the part corresponding to the primers and the final sequences were, respectively, 826 and 658 bp long. In both cases, the first codon of the amino acid chain corresponded to the second site, so the first nucleotide was also trimmed and the analyses were conducted on fragments of, respectively, 825 and 657 bp, in order to apply a model that allows for different substitution probabilities according to the position of the nucleotide in the codon. All the sequences used for the phylogenetic analysis were deposited in GenBank (GenBank codes are listed below).

Pairwise distance was calculated with MEGA-X, implementing Tamura-Nei substitution model (Tamura & Nei 1993) with gamma-distributed rates between sites.

Bayesian Inference was estimated using MrBayes 3.2 (Ronquist et al. 2009). The sequences of the specimens of *Dichotrachelus meregallii* and *D. ulbrichi* were added to the dataset used for inferring phylogeny of the *Dichotrachelus* by Meregalli et al. (2018) for the Jerry/Pat primers. Two runs with 4 chains were run for 2 million generations, sampling every 500 generations. The chains were left free to sample all the models of the GTR family using reversible jump Monte Carlo Markov Chain (MCMC) (Huelsenbeck et al. 2004). Heterogeneity of substitution rates among different sites was modelled with a 4-categories discretized Γ distribution, with a proportion of invariable sites. The matrix was partitioned so that substitution rates could vary according to the nucleotide position in the codon. The first 25 % generations were discarded (burn-in) and convergence was evaluated with the average standard deviation of split frequencies. Goodness of mixing was assessed looking at the acceptance rate of swaps between adjacent chains, following Ronquist et al. (2009). After a first analysis, temperature was lowered to 0.05 in order to improve swaps between chains. The resulting consensus tree was examined with Figtree (Rambaut 2014).

3. Results

3.1. Morphological analysis.

There are no constant morphological characters that allow differentiation of the western and eastern populations.

3.2. Molecular analysis.

3.2.1. Folmer primer.

All the specimens from Solda had identical sequences. The specimens from Gran Piano also had identical sequences, but one individual showed a possible polymorphism in five sites [(double peaks in the chromatogram for C/T (4 sites) and A/G (1 site), in two independent amplifications and sequencings]; in two of these sites (one C/T and the A/G) one of the alternative nucleotides was that present in the Solda specimens. Among all the specimens, 641 sites out of 657 were conserved (97.5 %), with a p-distance of 0.024 between the western and the eastern population. All substitutions, but one, are synonymous; the only different amino acid is a Valine replacing an Isoleucine in position 67 of the 219 amino acids chain in the western specimens.

3.2.2. Jerry/Pat primers.

No intrapopulation variation was detected among the specimens of each of the two populations; 813 sites out of 825 were conserved between the two populations (98.5 %), with a p-distance of 0.017; all substitutions are synonymous.

4. Discussion

4.1. Taxonomy

The discovery of the eastern population was surprising, since it was thought that *Dichotrachelus meregallii* was an endemite of the Western Alps. Usually, species of *Dichotrachelus* have a rather restricted range, even though *D. stierlini* has a wide distribution in the Eastern and Central Alps and reappears in the Western Alps with a slightly differentiated form, presently considered at subspecific rank, *D. stierlini knechti* Stierlin, 1875 (Alonso-Zarazaga et al. 2017), but whose taxonomic status has not yet been evaluated with a molecular analysis.

The two populations of *Dichotrachelus meregallii* cannot be distinguished morphologically. The limited differences in the mitochondrial sequences, in any case, do not exceed the range of intraspecific variation. In some species of *Dichotrachelus* no variation was observed in sympatric specimens (Meregalli et al. 2013 for *D. bischoffi* and *D. stierlini knechti*), and in the relatively widespread *D. maculosus* Fairmaire 1869 the populations from the extremes of the range, respectively Switzerland and the Maritime Alps, have a p-distance of 0.028 (Meregalli et al. 2018).

Based on the reciprocal isolation, a subspecific differentiation might be considered. An analysis of the concept and application of the subspecies rank is beyond the scope of this paper. Shortly, we preferred to avoid the use of an infraspecific taxon in part because this rank has no place in a phylogenetic classification and because of uncertainties in determining an objective threshold between species and subspecies ranks (Patten 2015). Therefore, in our opinion the combination of the morphological and molecular data indicates that the two populations of *D. meregallii* cannot be taxonomically differentiated.

4.2. Biogeographical remarks

The molecular differences between the two populations, limited but clear, indicate that the possibility of an anthropogenic introduction can be ruled out. This would have been in any case extremely unlikely, since these weevils occupy a niche, mosses on rocks, that have no significant chances to be accidentally transported from a fully natural site, isolated from any anthropic environment, to another equally natural site (see 4.4, Bionomy).

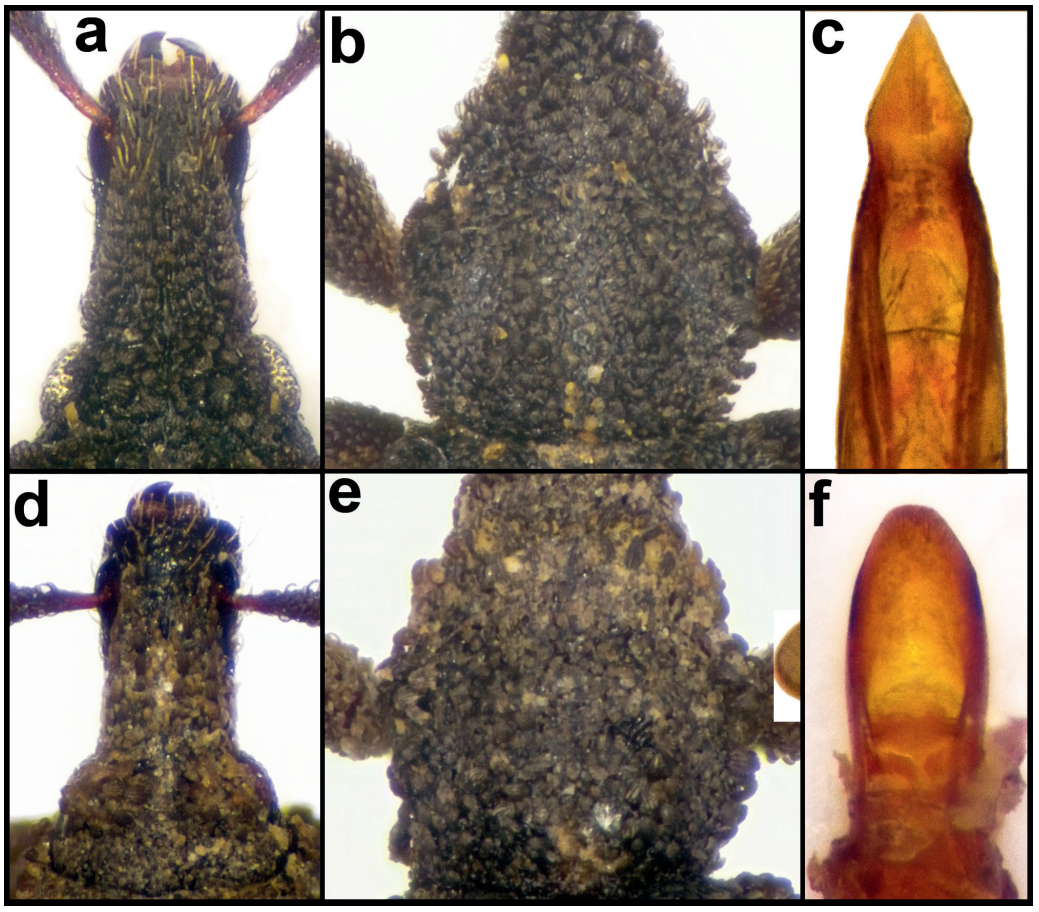

Fig. 2: a–c. *Dichotrachelus meregallii*, Solda. Rostrum, pronotum, apex of penis. **d–f.** *Dichotrachelus stierlini*, Val Martello. Rostrum, pronotum, apex of penis.

The disjunct distribution appears therefore to have been determined by natural causes, the most likely being the Pleistocene climatic oscillations that caused the fragmentation of the range of a species previously distributed along the entire alpine chain. As previously mentioned, a similar disjunct distribution occurs in another species of the genus *Dichotrachelus*, *D. stierlini*, even though the latter is quite broadly distributed in the Central-Eastern Alps. A similar disjunction was documented also for other alpine taxa, such as *Aeropedellus variegatus* (Fischer von Waldheim, 1846) (Orthoptera), that shares with the species of *Dichotrachelus* the presence in the Western and Eastern Alps and the absence from the central part of the chain, even though its eco-ethology is completely different, being a species associated with alpine meadows, that often occurs in a high number of individuals (Kirschner et al. 2020) and has a much higher individual vagility. *Dichotrachelus* have a particularly scarce capability of recolonization and range expansion, excepting at a very short distance, so their relict presence can give also information on the impact of the climatic oscillations on the alpine fauna in the various parts of the mountains. However, the presence of other isolated relict populations between the eastern and the western ones cannot be excluded. After all, the newly found specimens of southern Tyrol remained well hidden until 2015 and, moreover, the first specimen was found by chance.

4.3. Comparative remarks between *Dichotrachelus meregallii* and *D. stierlini*

In view of the new knowledge, specimens from the Central and Eastern Alps, originally referred to *Dichotrachelus stierlini* because of the broadly lobed tarsal segment 3, should be checked for possible reidentification as *D. meregallii*.

The two species are relatively similar, and differences may be overlooked at a first glance. *Dichotrachelus stierlini* has dorsal sides of rostrum feebly narrowed anteriad, ratio width at base / width between antennae 1.3 (sides of rostrum distinctly linearly

narrowed anteriad, ratio width at base / width between antennae 1.6 in *D. meregallii*), eyes flat (eyes prominent laterally), head lacking setae (head with dense setae), pronotum with sides scarcely broadened, moderately narrowed anteriad and with large erect setae in dorso-lateral part, usually denser in a tuft slightly beyond midlength (pronotum with sides distinctly broadened, strongly narrowed anteriad and with uniform vestiture of small setae on dorso-lateral part), apex of penis completely different (Fig. 2).

4.4. Bionomy

Dichotrachelus meregallii colonizes small cushion-shaped mosses on exposed rocks in the alpine and the snow zones (Fig. 1, b–d). The western population is also present in the low-montane zone in deciduous forest habitat, again where mosses on rocks are colonized. All specimens were found on siliceous rocks. At high altitude, the adults hatch in early summer and, since young larvae were present when the pupae were seen, the pre-imaginal life cycle appears to require two years. Pupae of the specimens of the western population from the habitat at low altitude were found in late August (Meregalli, personal observation). This species, in all life stages, feeds on mosses (Meregalli et al. 2018).

4.5. Distribution

4.5.1. Western population.

The southernmost specimens are present on the northern side of the Susa Valley, on Mount Tabor, near Bardonecchia. From there, *Dichotrachelus meregallii* is present on the higher parts of the Lanzo Valleys, where it reaches the lowest altitude at 900 m, near the village of Ceres, in the lower montane zone. In the Orco Valley it was found at Col del Nivolet, at the head of the valley, and also on the southern slopes of the Gran Paradiso massif, its northernmost habitat (Meregalli, unpublished data). In the Orco Valley it is sympatric, but not exactly syntopic, with *D. stierlini knechti* which, however, prefers mosses amidst gravels and small stones, whereas *D. meregallii* is usually on exposed mosses on large boulders.

4.5.2. Eastern population.

The eastern population was found in two different sites, a few km distant, above Solda (Sulden) in South Tyrol. Also here *Dichotrachelus meregallii* is sympatric with *D. stierlini*, in this case *D. stierlini stierlini* but, as in the Western Alps, the two species occupy different niches.

4.6. Conservation.

There does not appear to be any risk for the western population since this has a relatively wide geographical range and, at least occasionally, has a broad altitudinal range. Different considerations apply to the eastern population, which is fragmented in two areas of apparently very limited extension. These sites could easily be damaged, either by human impact (new ski resorts), or by ecological variations following global climate change. So this population can be considered as **vulnerable.**
After acceptance of the manuscript another finding of the eastern population of *Dichotrachelus meregallii* became known: Sulden Valley, Razoi, scree slope (46°33'40"N 10°36'33"E, 2700 m) 7 specimens, 22.7.2021, leg. M. Kahlen.

5. *Dichotrachelus ulbrichi* Osella, 1977

One more species needs to be discussed, *Dichotrachelus ulbrichi* Osella, 1977.
Osella (1977), when describing this species, based on the male holotype only, suggested that it was related to *D. meregallii*, and proposed a *D. meregallii* species-group for *D. meregallii* and *D. ulbrichi*. According to this author, the group would occupy an intermediate position between the *D. rudeni* Stierlin, 1853 and the *D. stierlini* groups. *D. ulbrichi* has never been collected since. In view of the new records of *D. meregallii* from the eastern Alps, its study was important to confirm the hypothetical relationships.

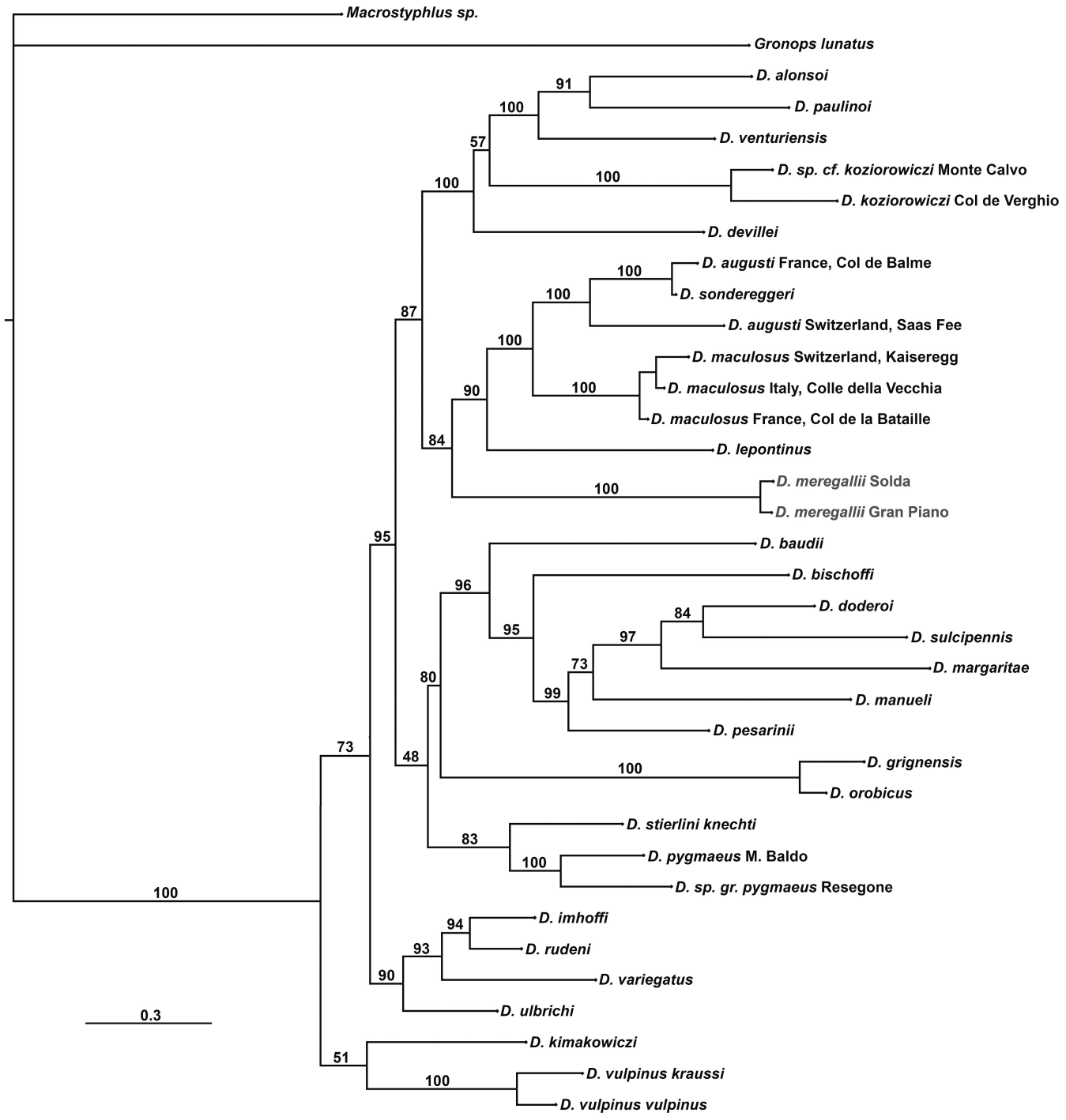

Fig. 3: Bayesian Inference consensus tree based on mt-CoxI of the relationships among several species of *Dichotrachelus*, with emphasis on *Dichotrachelus meregallii*. Branch post probability support is indicated on the branches, in percentage. Scale bar unit: expected substitutions per site.

Two of the authors (M.M., M.K.) organized a specific search at the type locality, the Kärntner Storschitz, near Eisenkappel, at the border between Austria and Slovenia. No adults were found, but luckily a single larva, after a very long search, was collected inside a moss on a stone near the top of the mountain. No other species of moss-associated *Dichotrachelus* are known from the entire region, therefore we are confident that the larva was correctly referred to *D. ulbrichi*.

According to the Bayesian inference based on its mt-CoxI sequence (Fig. 3), *Dichotrachelus ulbrichi* is not closely related to *D. meregallii*. Rather, it belongs to the *D. rudeni* group. This affinity is also indicated by the shape of the apex of the penis, which has minute lateral spiniform expansions similar to those of *D. rudeni* and related species

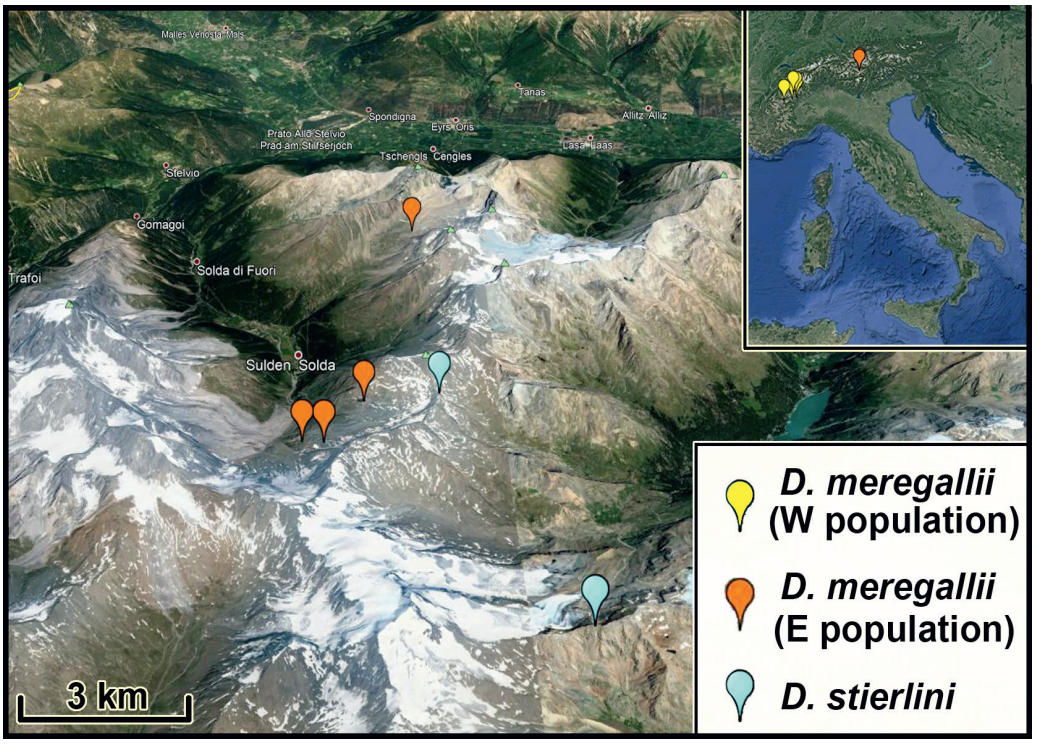

Fig. 4: *Dichotrachelus meregallii*, distribution map. Two localities of *D. stierlini* from Val Martello are also reported. Map data: Google Earth, Maxar Technologies, used according to Google Earth Terms of Service.

[see figures 1–3 in Osella 1977 for *D. ulbrichi* and tav. V, fig. 1 in Osella 1968 for *D. rudeni*), and differs in the apex being more prominent anteriad, but rounded, not acutely pointed as in *D. meregallii*.

In the Bayesian analysis (Fig. 3) *Dichotrachelus meregallii* clustered in a clade comprising a few other species from the Alps, namely those of the *D. maculosus* Fairmaire, 1969 group and *D. lepontinus* Osella, 1971, as sister to these species, with good support (84% post probability). Morphologically, however, it differs from the others by the shape of the genitalia, the broadened lobes of tarsomere 3, and the shape of the elytra. Its inclusion in a distinct species-group, as proposed by Osella (1977), is justified.

Riassunto

Il ritrovamento di una popolazione isolata di *Dichotrachelus meregallii* nelle Alpi orientali, in Tirolo meridionale, ha stimolato uno studio su base morfologica e molecolare per paragonare i nuovi reperti con la popolazione tipica delle Alpi occidentali. I risultati indicano che le due popolazioni differiscono soltanto per minime variazioni della sequenza dei geni mitocondriali, che non supportano una differenziazione tassonomica. I dati suggeriscono che la specie ebbe una diffusione continua su tutto l'arco alpino, e che la sua attuale distribuzione relitta fu probabilmente determinata dalle variazioni climatiche pleistoceniche. Le relazioni filogenetiche di *D. ulbrichi*, originariamente associato al gruppo di *D. meregallii,* sono state approfondite. Sulla base della sequenza dei geni mitocondriali, la specie risulta appartenere al gruppo di *D. rudeni.*

Acknowledgments

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Contribution to the paper: M.M. and M.K: general structuring of the research, field sampling and preparation of the paper; M.M.: phylogenetic analyses, discussion; A.S.: DNA extraction, laboratory work.

GenBank codes

MW649095.1. *Dichotrachelus meregallii* Gran Piano, specimen 1, Folmer primers
MW649092.1. *Dichotrachelus meregallii* Solda, specimen 1, Folmer primers
MW649094.1. *Dichotrachelus meregallii* Solda, specimen 2, Folmer primers
MW649089.1. *Dichotrachelus meregallii* Gran Piano specimen 1, Jerry/Pat primers
MW649089.1. *Dichotrachelus meregallii* Gran Piano specimen 2, Jerry/Pat primers
MW649091.1. *Dichotrachelus meregallii* Gran Piano, specimen 3, Jerry/Pat primers
MW649090.1. *Dichotrachelus meregallii* Solda, specimen 1, Jerry/Pat primers
MW649093.1. *Dichotrachelus meregallii* Solda, specimen 2, Jerry/Pat primers
MW602206.1. *Dichotrachelus ulbrichi*, Folmer primers
MW602205.1. *Dichotrachelus ulbrichi*, Jerry/Pat primers

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