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Phylogeny of the *Saxifraga*-associated species of *Dichotrachelus* (Insecta: Coleoptera: Curculionidae), with remarks on their radiation in the Alps

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Abstract

A phylogenetic analysis of the *Saxifraga*-associated species of *Dichotrachelus* was carried out based on morphological and, for some species, molecular data. The various methods implemented gave congruent results and yielded a significantly supported phylogenetic inference of the relationships among the species. *Saxifraga*-associated species form a monophyletic unit, and some species-groups were evidenced, distributed in the eastern, central and western Alps, respectively. Based on the phylogenetic inference, and taking into account palaeogeological and palaeogeographical information, a biogeographic scenario of the events that may have led to the present-day distribution is proposed.

Key words

Alpine speciation, ecological niche-shift, Cyclominae, *Dichotrachelus*, glacial refugia, COI sequences, phylogeography, taxonomy.

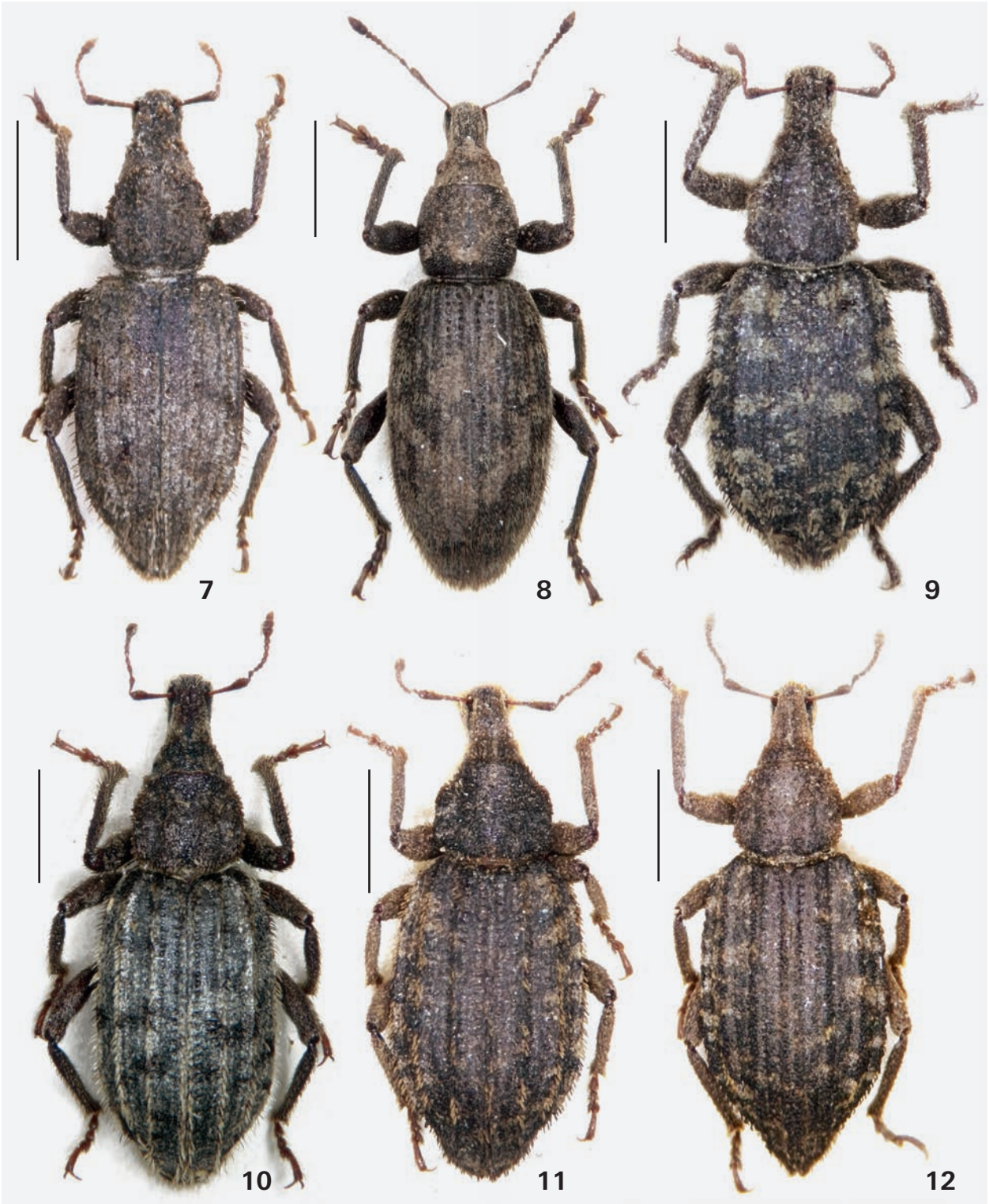
1. Introduction

Dichotrachelus Stierlin, 1853 is a genus of sexually reproducing, apterous weevils (Figs. 1–12) composed of 59 species native to southern Europe (56 species) and North Africa (3 species) (Meregalli 2013). It was only recently referred to the subfamily Cyclominae Schoenherr, 1826 (Alonso-Zarazaga & Lyal 1999), tribe Dichotrachelini Hoffmann, 1957. Within the subfamily its relationships are unknown (Oberprieler 2010) and no sister group has been recognized. The genus was revised by Osella (1968, 1971), and descriptions of new species or rearrangements of known species were subsequently published (Meregalli 1983a, 1985, 1987, 1989, 1992; Osella et al. 1983; Meregalli & Osella 1975; 2007; Germann & Baur 2010).

Two major species-complexes can be recognized within *Dichotrachelus*, differentiated in their morphology and biology. The first complex includes 15 "large-sized" species relatively homogeneous in morphology, at least 5.5 mm long (including rostrum), native to the Alps and the Pyrenees, where they live at high altitudes; these species feed on various species of *Saxifraga* (Saxifragaceae; Meregalli 1980), with the notable exception of *D. imhoffi* Stierlin, 1857, associated with mosses (Germann & Baur 2010). This complex includes the

Figs. 1–6. Species of *Dichotrachelus*. **1**: *D. stierlini*, Colle dell'Arietta. **2**: *D. rudeni*, Col de Balme. **3**: *D. imhoffi*, Val Seriana. **4**: *D. luzei*, Mt. Grintovec. **5**: *D. kahleni*, Mt. Sernio (paratypus). **6**: *D. philippi* cf., Mt. Baldo. Bar: 2 mm.

type species, *Dichotrachelus sulcipennis* Stierlin, 1853. The second complex includes the remaining 44 species, which are "small-sized", less than 5 mm long, live on mosses (Bryophyta, often, but not exclusively, of the genus *Grimmia* Hedw.; Meregalli & Osella 1975), at medium to high altitude, in forest habitats and high altitude coenoses of the Alps and the Pyrenees, Transylvania, central and southern Apennines, Corsica, Sardinia, Sicily, southern France, Iberian peninsula, Morocco and Algeria. This second complex is more heterogeneous in morphology and includes several apparently monophyletic subunits, or species-groups, in part discussed by Meregalli (1987).



Figs. 7–12. Species of *Dichotrachelus*. **7**: *D. grignensis*, Mt. Grigna. **8**: *D. baudii*, Cima Ciantiplagna. **9**: *D. bischoffi*, Val Clavalité. **10**: *D. manueli*, Mt. Rocciamelone. **11**: *D. sulcipennis pedemontanus*, Moncenisio. **12**: *D. linderi*, Mt. Canigou. Bar: 2 mm.

Due to the often very small distribution ranges of the species and their limited dispersal ability, *Dichotrachelus* has been considered a useful taxon for inferences of historical biogeography (Vigna-Taglianti 1968; Meregalli 1987). However, most of the studies so far carried out were strictly taxonomic contributions. Historical biogeography was briefly discussed by Osella (1968, 1971), and more deeply investigated only for the Iberian and North-African species (Meregalli 1987). The results are briefly summarized here.

As indicated by Meregalli (1987) on the basis of the present-day distribution of the species-groups, the genus *Dichotrachelus* differentiated in territories surrounding the western Tethys basin in the late Oligocene or

early Miocene, apparently before or soon after the lands started to break into microplates. In the structure of the genital sclerite, some of the small-sized alpine species share similarities with *D. ribesi* Gonzalez, 1964 native to north-east Spain and *D. berberus* Meregalli, 1989 from North Africa – thus those at the opposite extremes of the Miocene Alpidian chain (see Meregalli 1987: figs. 249, 250). These two small-sized species were considered to be very old relicts (Meregalli 1989). Considering the poor dispersal capability of the *Dichotrachelus*, probably preventing them from crossing geographic barriers, such a distribution of apparently related species suggests that disjunction of the lineages originated by vicariance, and occurred when the Alpidian chain started to fragment and the small tectonic plates started their migration through the west Mediterranean basin. Several different palinspastic models of the palaeogeography of the Western Tethys are available (Rögl 1998; Popov et al. 2004; Harzhauser & Piller 2007; and more), but all agree that the fragmentation of these territories had already occurred in the early Miocene.

Other species-groups of small-sized *Dichotrachelus* are present in southern Spain and northern Africa (*D. baeticus* Meregalli, 1987 and *D. rifensis* Meregalli, 1983; *D. deferreri* Meregalli & Alonso-Zarazaga, 1988 and *D. afer* Peyerimhoff, 1915), Corsica and Sardinia (*D. koziorowiczi* Desbrochers des Lodges, 1873 and *D. sardous* A. & F. Solari, 1903), the Pyrenees and the Cantabrian Mountains (*D. pyrenaicus* Osella, 1971 and *D. cantabricus* Franz, 1954), the Alps and the Apennines (several species of the *D. rudeni* Stierlin, 1853, *D. maculosus* Fairmaire, 1869 and *D. stierlini* Gredler, 1856 groups); another species-group includes several taxa, confined to the Iberian Peninsula and to some isolated massifs of southern France, that present a relatively uniform structure of the genital sclerite (see Meregalli 1987: figs. 252–271, 273, 274).

The lineage of the alpine large-sized species underwent a niche-shift from mosses to *Saxifraga* as the host-plants. A niche-shift consisting of an adaptation to a "new" host-plant, generally already used as a refuge by species associated with other host-plants, was discussed by Colonnelli & Osella (1998), who suggested that such a niche-shift could be fostered in conditions of environmental stress. Niche-shift has even been associated with the major differentiation of phylogenetic lineages of the families of Curculionoidea (Marvaldi et al. 2002). In the case of *Dichotrachelus*, the species of *Saxifraga* on which these weevils develop are typical of rocky crevices, and are generally syntopic with mosses. These habitats are often subject to strong and rapid microclimate variation and experience more extreme conditions of humidity and temperature compared with mosses on rocks in forests. *Saxifraga* roots maintain more stable conditions, and may have been used primarily as refuge plants – this still occurs nowadays for some of the small-sized species, whose larval stages develop on mosses but whose imagoes are occasionally found, in the coenoses of higher altitude, amidst the roots of *Saxifraga*. This has even led to some citations of *Saxifraga* as being the host-plant of some of the small-sized *Dichotrachelus*, i.e., *D. maculosus* (Osella 1968: p. 418; Meregalli 1980: p. 127; both as "*D. alpestris*") and *D. rudeni* (Meregalli 1980: p. 127). In both cases, however, the data were based on imagoes having been found underneath plants of *Saxifraga*, or below small stones standing over saxifrages, whereas larval stages of the two species were exclusively found in mosses (Germann 2011; Meregalli, personal observation). Adaptation of these low-vagility insects to species of *Saxifraga* requiring low temperature and now restricted – in the Alps and Pyrenees – to high altitudes boosted their speciation and modeled their present-day distribution.

No phylogenetic analysis, either molecular or morphological, has ever been attempted for the genus *Dichotrachelus*. In this work we conduct a phylogenetic analysis of the large-sized, *Saxifraga*-associated species. Our intent is to test the validity of the present taxonomic framework, to infer evolutionary relationships among the species, and to suggest some hypotheses on speciation events that led to the present-day distribution. The study was mainly based on a morphological dataset, but it was possible to include molecular data for some of the species.

2. Materials and methods

2.1. Sample origin

***Dichotrachelus* taxa.** A total of 302 specimens of 20 species and subspecies (15 large-sized and 5 small-sized ones, some shown in Figs. 1–12) from 31 localities were used for the morphological analysis (see Table 1 for details). 36 specimens of 8 species (6 large-sized and 2 small-sized ones) from 13 localities were used for sequencing of COI (see Table 3 for details), but part of the sequences had to be excluded prior to the molecular analysis (see section 2.3.). Specimens for molecular analysis were sampled by the authors in summer 2011; one specimen of *Dichotrachelus maculosus* from Switzerland was kindly provided by Christoph Germann. The weevils were hand-collected, immediately killed in 95% ethanol and preserved at –20°C. Before grinding them, almost all specimens used for the molecular analysis were used also for the morphological matrix, together with specimens from Meregalli's collection.

Outgroup taxa for morphological analysis. It is difficult to select species of other genera of Cyclominae as outgroup taxa for morphology, since the genus-rank relationships of *Dichotrachelus* are unknown (Oberprieler 2010) and the genus altogether appears quite isolated. More importantly, however, for many of the non-genital

Table 1. Populations and specimens used in morphological analysis. Column "Locality & sample": Samples marked with "C" were collected in summer 2011, the others are from Meregalli's collection (if both sources apply to a particular locality, they are given in two separate lines). Within each species or, if applicable, subspecies, some collecting localities are close to each other (Lago Brocan and Colle di Fremamorta for *D. doderoi*; Monte Rocciamelone and Malciaussia for *D. manueli*, Colle della Vecchia and Cima Ciantiplagna for *D. baudii* and *D. margaritae*, etc.); different samples from the same or from closely neighbouring localities were combined into a single line of the morphological matrix, the combined samples bear the same elevated number at the end. Populations indicated with ** were examined, and in some cases used for the photographs, but not used for the matrix of characters. Column "Fig.": The numbers refer to the localities (or groups of closely neighbouring localities) as plotted in Figs. 58 (E#) and 59 (W#), showing the eastern resp. western part of the distribution area. Column "Char." gives the basic characterization of species: before dash la = large-sized, sm = small-sized; after dash sa = *Saxifraga*-associated, mo = moss-associated. The remaining colums give the number of specimens that were altogether used or in which specific body parts were studied.

Locality & Sample	Fig.	Species & Subspecies	Char.	N. specimens	N. specimens in ventral view	N. male genitalia	N. male genital sclerites	N. female genitalia
Colle del Nivolet 2800 m C	W6	*D. sulcipennis pedemontanus* Stierlin, 1878	la-sa	**9**	**9**	**1**	**1**	**2**
Col d'Iseran (F) 2500 m	W7	*D. sulcipennis pedemontanus* Stierlin, 1878	la-sa	**14**	**2**	**2**	**1**	**1**
Pian della Mussa 2700 m	W8	*D. sulcipennis pedemontanus* Stierlin, 1878	la-sa	**28**	**3**	**6**	**1**	**1**
Colle del Moncenisio	W10	*D. sulcipennis pedemontanus* Stierlin, 1878	la-sa	**8**	**2**	**1**	**1**	**2**
Val Soana, Piamprato 1500 m	W4	*D. sulcipennis bernhardinus* Stierlin, 1878	la-sa	**8**	**4**	**3**	**1**	**3**
Col Ferret 2200 m	W2	*D. sulcipennis bernhardinus* Stierlin, 1878	la-sa	**12**	**1**	**2**	**1**	**1**
Col d'Olen 2900 m [1]	W1	*D. sulcipennis sulcipennis* Stierlin, 1853	la-sa	**3**	**1**	**1**	**1**	**1**
Col d'Olen 2900 m C [1]	W1	*D. sulcipennis sulcipennis* Stierlin, 1853	la-sa	**6**	**6**	**1**	**1**	**0**
Colle dell'Agnello 2800 m	W12	*D. margaritae* Osella, 1968	la-sa	**5**	**2**	**1**	**1**	**1**
Cima Ciantiplagna 2600 m [2]	W11	*D. margaritae* Osella, 1968	la-sa	**22**	**2**	**3**	**1**	**1**
Colle della Vecchia 2600 m C [2]	W11	*D. margaritae* Osella, 1968	la-sa	**6**	**6**	**3**	**1**	**1**
Argentera, Lago Brocan 2000 m [3]	W14	*D. doderoi doderoi* A. & F. Solari, 1905	la-sa	**2**	**1**	**1**	**1**	**1**
Colle di Fremamorta 2600 m C [3]	W14	*D. doderoi doderoi* A. & F. Solari, 1905	la-sa	**5**	**5**	**1**	**0**	**1**
Colle del Mulo 2000 m [4]	W13	*D. doderoi vignai* Osella, 1968	la-sa	**7**	**0**	**3**	**1**	**2**
Colle del Mulo 2500 m C [4]	W13	*D. doderoi vignai* Osella, 1968	la-sa	**2**	**2**	**1**	**0**	**1**
Mont Canigou (F) 2750m	—	*D. linderi* Fairmaire, 1852	la-sa	**4**	**0**	**1**	**1**	**2**
Malciaussia 2600 m [5]	W9	*D. manueli* Marseul, 1871	la-sa	**14**	**4**	**3**	**1**	**3**
Monte Rocciamelone 3200 m [5]	W9	*D. manueli* Marseul, 1871	la-sa	**2**	**1**	**0**	**0**	**1**
Monte Rocciamelone 2900 m C [5]	W9	*D. manueli* Marseul, 1871	la-sa	**6**	**6**	**1**	**1**	**1**
Val Clavalité 2800 m	W3	*D. bischoffi* Stierlin, 1878	la-sa	**11**	**2**	**2**	**2**	**2**
Colle Bardoney 2900 m C	W5	*D. bischoffi* Stierlin, 1878	la-sa	**9**	**9**	**4**	**1**	**2**
Colle della Vecchia 2600 m [6]	W11	*D. baudii* Seidlitz, 1875	la-sa	**13**	**1**	**4**	**2**	**2**
Colle della Vecchia 2600 m C [6]	W11	*D. baudii* Seidlitz, 1875	la-sa	**3**	**3**	**1**	**1**	**1**
Cima Ciantiplagna 2600 m [6]	W11	*D. baudii* Seidlitz, 1875	la-sa	**51**	**1**	**1**	**0**	**0**
Colle Basset 2400 m [6]	W11	*D. baudii* Seidlitz, 1875	la-sa	**4**	**0**	**0**	**0**	**0**
Colle dell'Agnello 2800 m [7]	W12	*D. baudii* Seidlitz, 1875	la-sa	**9**	**0**	**1**	**1**	**1**
Monte Maniglia 2900 m C [7]	W12	*D. baudii* Seidlitz, 1875	la-sa	**4**	**4**	**0**	**0**	**0**
Massiccio della Grigna	E1	*D. grignensis* Breit, 1902	la-sa	**2**	**2**	**1**	**1**	**1**
Pizzo Arera**	E2	*D.* cf. *grignensis* Breit, 1902	la-sa	**1**	**1**	**1**	**1**	**0**
Monte Pasubio	E4	*D. philippi* Osella, Bellò & Pogliano, 1983	la-sa	**2**	**1**	**0**	**1**	**1**
Monte Baldo, Cima Valdritta**	E3	*D.* cf. *philippi* Osella, Bellò & Pogliano, 1983	la-sa	**1**	**1**	**1**	**1**	**0**
Grintovec (SL) 1800 m	E7	*D. luzei* Ganglbauer, 1895	la-sa	**3**	**0**	**2**	**2**	**1**
Monte Sernio 1730 m	E5	*D. kahleni* Meregalli & Osella, 2007	la-sa	**4**	**3**	**1**	**1**	**2**
Monte Plauris**	E6	*D.* cf. *kahleni* Meregalli & Osella, 2007	la-sa	**1**	**1**	**1**	**0**	**0**
Passo dello Spluga	E8	*D. imhoffi* Stierlin, 1857	la-mo	**2**	**2**	**1**	**1**	**1**
Colle Bardoney 2800 m C	W5	*D. stierlini* Gredler, 1856	sm-mo	**5**	**5**	**2**	**1**	**1**
Colle della Vecchia 2600 m C	W11	*D. maculosus* Fairmaire, 1869	sm-mo	**3**	**3**	**1**	**1**	**2**
Col de Balme (CH) 2700 m	W15	*D. rudeni* Stierlin, 1853	sm-mo	**3**	**3**	**1**	**1**	**1**
Sierra de Queija (SP)	—	*D. laurae galicianus* Meregalli, 1987	sm-mo	**4**	**4**	**2**	**2**	**2**
Djebel Tazzeka (MO)	—	*D. berberus* Meregalli, 1989	sm-mo	**4**	**4**	**2**	**2**	**2**
		Total:		**302**	**107**	**64**	**38**	**48**

and all genital characters used herein to infer the phylogeny of *Dichotrachelus* no homologies can be found in other genera of the subfamily. In particular, the structure of the male genital sclerites is exclusive for the genus among all weevils, and also the shape of female sternite VIII is completely different in *Dichotrachelus* from all

other Cyclominae. In addition, other body characters such as the shape of the pronotum are too strongly homoplastic among Curculionidae and even Cyclominae to let scoring of non-*Dichotrachelus* outgroup taxa appear useful. It appeared to be the only presently viable procedure to define the five included small-sized species, belonging to different morphological groups (as proposed by OSELLA 1968 and MEREGALLI 1987), as outgroup taxa: *D. maculosus*, *D. stierlini* Gredler, 1856, *D. berberus*, *D. laurae galicianus* Meregalli, 1987 and *D. rudeni*. *Dichotrachelus berberus* was chosen as the most distant outgroup taxon since it has been considered to be a relict species, possibly belonging to one of the earlier derived lineages of the genus (MEREGALLI 1989). We are aware that this solution is preliminary and leads to some uncertainty in the placement of the root in our phylogenetic trees.

Outgroup taxa for molecular analysis. COI sequences of three Cyclominae species were kindly provided by Rolf Oberprieler (CSIRO, Australia) and these were used as outgroup data: *Aoplocnemis rufipes* Bohemann, 1845, *Pelolorhinus variegatus* (Fåhraeus, 1842) and *Steriphus major* (Blackburn, 1890). COI sequences of 159 further species of Curculionoidea were retrieved from GenBank and were used to gather some basic information on the position of the genus *Dichotrachelus* within the Curculionoidea.

2.2. Morphological data

Morphological characters were selected in part based on literature, in particular MEREGALLI (1983b, 1985), and in part based on new observations. We selected only the characters that among the species subject to this study occur in conditions sufficiently different for defining states. Several additional characters show different conditions among the small-sized, moss-associated species, but do not vary (or are not present) in the *Saxifraga*-associated species. These characters were not taken into account. Coding was conducted in a discrete quantitative way as much as possible, based on direct observation under the stereomicroscope and on photographs of the morphological structures. Genitalia were dissected manually after boiling the entire abdomen in 10% KOH. Aedeagi were observed dry, female sternite VIII and the aedeagal genital sclerite were observed in 90% glycerol. Photographs were taken with a Nikon Coolpix P6000 camera mounted on a Leica S6E stereomicroscope. A series of photographs at different focus planes were taken and the stack was combined to a single image with Zerene Stacker 1.04 (Zerene Systems LCC). The final images were processed using Photoshop CS3 (Adobe Systems Inc.).

We used 44 discrete morphological characters, 23 based on external morphology and 21 based on genitalia, 9 of the latter being pertinent to the male genital sclerite (Figs. 13–38). Terminology mainly follows LYAL (2013). It could be suggested that the contribution of the male genital sclerite to the analysis outweighs that of the other characters, so that the topology of the resulting trees may have been driven by the shape of this sclerite. The phylogenetic significance of insect genitalia has often been discussed and it was suggested that, since the genitalia are under sexual selection and therefore the rate of their evolution is extremely rapid, there may not be observable phylogenetic signal left in the structures (LOSOS 1999). However, a recent survey demonstrated that genitalia do indeed possess at least the same phylogenetic signal as non-genital structures (SONG & BUCHELI 2010). An especially high content of phylogenetic information of the male genitalia has also been demonstrated for e.g. Dictyoptera (KLASS 1997; KLASS & MEIER 2006), where genital morphology and molecular-based results show congruence in the delimitation of the major lineages (DJERNÆS et al. 2012). To test the phylogenetic signal of the genital vs. the non-genital characters in *Dichotrachelus*, we have analysed independently the 23 characters for non-genital structures and the 21 characters based on genitalia. One character regarding foodplants (mosses or *Saxifraga*) was added since host-plant associations in weevils are considered to reflect phylogenetic lineages (MARVALDI et al. 2002). All characters were treated as not ordered. See Table 2 for the matrix with the character scores.

1. Body length including rostrum: 0 = < 5.5 mm; 1 = > 5.5 mm.
2. Median keel of rostrum (rostrum: Figs. 13–16): 0 = absent; 1 = present (Fig. 16).
3. Vestiture of scales on dorsum of rostrum beyond midlength: 0 = present on both median and lateral parts (Fig. 14); 1 = absent from median part but present on lateral parts (Fig. 16); 2 = absent on all parts (Fig. 13).
4. Ratio length/width of rostrum (length measured from tip of rostrum between mandible bases to level of anterior margin of eyes; width measured at anterior margin of eyes): 0 = long and narrow, ratio l/w ca. 3; 1 = short and wide, ratio l/w ca. 2.
5. Erectedness of setae on rostrum: 0 = not erect; 1 = erect on the whole surface; 2 = erect only along rostral margin.
6. Thickness of setae on rostrum: 0 = thick; 1 = thin.
7. Ratio length/width of pronotum (pronotum: Figs. 20–24): 0 = strongly transverse, ratio l/w 0.7–0.8; 1 = weakly transverse, ratio l/w ca. 0.9; 2 = as long as wide; 3 = longer than wide.
8. Sides of pronotum: 0 = nearly parallel in its whole length; 1 = maximum width in proximal third, converging anteriad in middle and distal thirds; 2 = angularly broadened in its middle third, and equally converging posteriad and anteriad; 3 = curvilinearly broadened in its middle third, more strongly converging anteriad in distal third than posteriad in proximal third.
9. Dorsolateral vertical grooves of pronotum: 0 = shallow; 1 = deep.

Table 2. Morphological character matrix.

Character number	**0 1**	**0 2**	**0 3**	**0 4**	**0 5**	**0 6**	**0 7**	**0 8**	**0 9**	**1 0**	**1 1**	**1 2**	**1 3**	**1 4**	**1 5**	**1 6**	**1 7**	**1 8**	**1 9**	**2 0**	**2 1**	**2 2**	**2 3**	**2 4**	**2 5**	**2 6**	**2 7**	**2 8**	**2 9**	**3 0**	**3 1**	**3 2**	**3 3**	**3 4**	**3 5**	**3 6**	**3 7**	**3 8**	**3 9**	**4 0**	**4 1**	**4 2**	**4 3**	**4 4**	**4 5**
berberus	0	0	2	0	1	1	3	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	3	2	2	0	1	0	0	2	0	1	0
*baudii*_Ciantiplagna	1	0	0	1	0	0	2	0	0	1	1	0	1	1	0	1	1	0	1	0	0	1	3	1	0	0	1	1	0	1	0	1	0	1	2	0	6	2	1	0	1	1	1	1	1
*baudii*_Agnello	1	0	0	1	0	0	2	0	0	1	1	0	1	1	0	1	1	0	1	0	0	1	3	1	0	0	1	1	0	1	0	1	0	1	2	0	6	2	1	0	1	1	1	1	1
*bischoffi*_Clavalité	1	0	0	1	1	0	3	0	1	1	1	0	2	1	0	1	1	1	0	0	0	0	2	0	1	0	0	0	0	1	0	1	0	1	2	0	6	2	1	0	1	1	1	1	1
*bischoffi*_Bardoney	1	0	0	1	1	0	3	0	1	1	1	0	2	1	0	1	1	1	0	0	0	0	2	0	1	0	0	0	0	1	0	1	0	1	2	0	6	2	1	0	1	1	1	1	1
*doderoi*_Mulo	1	0	0	1	2	0	2	3	0	2	2	1	2	1	0	1	1	0	0	0	0	0	2	1	0	1	0	1	0	1	0	0	0	2	4	0	7	6	1	1	0	0	0	2	1
*doderoi*_Argentera	1	0	0	1	2	0	2	3	0	2	3	1	2	1	0	1	1	0	0	0	0	1	2	1	0	1	0	1	0	1	0	0	0	2	4	0	7	6	1	1	0	0	0	2	1
grignensis	1	0	0	1	0	0	3	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0	1	1	0	6	2	1	0	1	0	1	1	1
imhoffi	0	0	0	1	1	0	3	0	0	0	0	0	1	1	1	0	1	0	0	0	0	2	1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	4	3	0	2	0	0	0	1	0
kahleni	1	0	0	1	0	0	3	0	0	0	2	0	1	1	0	0	1	0	0	0	0	1	2	0	0	0	0	0	0	1	0	0	0	0	1	0	1	3	4	1	0	1	0	0	1	1
laurae galicianus	0	0	2	0	1	1	3	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	2	0	0	0	0	0	0	0
linderi	1	0	0	1	2	0	2	3	1	1	2	1	2	0	0	1	0	0	0	0	1	1	2	1	0	1	0	1	0	1	0	0	1	2	4	0	7	6	1	1	0	0	0	1	1
luzei	1	0	0	1	0	0	3	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	1	2	0	0	0	0	0	0	1	0	0	0	1	1	1	5	4	1	0	1	0	0	1	1
maculosus	0	0	0	1	0	0	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	2	1	0	0	0	0	0	0	1	0
manueli	1	1	1	1	2	0	2	0	1	0	1	0	2	1	0	1	1	0	0	0	0	0	2	1	0	1	0	1	0	1	0	0	0	1	2	0	6	5	1	0	0	0	0	2	1
*margaritae*_Agnello	1	0	0	1	2	0	1	3	1	2	2	1	2	1	0	1	1	0	0	1	0	0	2	1	0	1	0	1	0	0	0	0	0	2	4	0	7	6	1	1	0	0	0	2	1
*margaritae*_Vecchia	1	0	0	1	2	0	1	3	1	2	2	1	2	1	0	1	1	1	0	1	0	0	2	1	0	1	0	1	0	0	0	0	0	2	4	0	7	6	1	1	0	0	0	2	1
philippi	1	0	0	1	0	0	3	0	1	0	2	0	1	1	0	1	1	0	0	0	0	1	2	0	0	0	0	0	0	1	0	0	0	1	1	0	5	4	1	0	1	0	0	1	1
rudeni	0	0	0	1	0	0	3	0	0	0	0	0	1	1	1	0	0	0	0	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	1	0	4	3	0	2	0	0	0	1	0
stierlini	0	0	0	1	0	0	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	0	2	1	0	0	0	0	0	0	1	0
*sulcipennis*_Iseran	1	0	0	1	2	0	1	1	1	2	3	1	2	1	0	1	1	0	0	1	0	0	2	1	0	1	0	1	0	1	1	0	0	2	4	0	7	7	1	1	0	0	0	2	1
*sulcipennis*_Soana	1	0	0	1	2	0	1	1	1	2	3	1	2	1	0	1	1	0	0	1	0	0	2	1	0	1	0	1	0	1	0	0	0	2	4	0	7	7	1	1	0	0	0	2	1
*sulcipennis*_Mussa	1	0	0	1	2	0	1	1	1	2	3	1	2	1	0	1	1	0	0	1	0	0	2	1	0	1	0	1	0	1	1	0	0	2	4	0	7	7	1	1	0	0	0	2	1
*sulcipennis*_Moncen	1	0	0	1	2	0	1	1	1	2	3	1	2	1	0	1	1	0	0	1	0	0	2	1	0	1	0	1	0	1	1	0	0	2	4	0	7	7	1	1	0	0	0	2	1
*sulcipennis*_Ferret	1	0	0	1	2	0	1	1	1	3	4	2	2	1	0	1	1	0	0	1	0	0	2	1	0	1	0	1	0	1	0	0	0	2	4	0	7	7	1	1	0	0	0	2	1
*sulcipennis*_Olen	1	0	0	1	2	0	0	1	1	3	4	1	2	1	0	1	1	0	0	1	0	0	2	1	0	1	0	1	0	1	0	0	0	2	4	0	7	7	1	1	0	0	0	2	1
*sulcipennis*_Nivolet	1	0	0	1	2	0	1	1	1	2	3	2	2	1	0	1	1	0	0	1	0	0	2	1	0	1	0	1	0	1	1	0	0	2	4	0	7	7	1	1	0	0	0	2	1

10. Ratio between maximum width of pronotum and maximum width of its median groove: 0 = > 2.6 (narrow groove); 1 = 2.6–2.5; 2 = 2.5–2.4; 3 = < 2.3 (broad groove).

11. Ratio length/width of pronotal groove, width measured in the point of maximum width of groove: 0 = > 2.6; 1 = 2.31–2.60; 2 = 2.00–2.30; 3 = 1.81–1.99; 4 = < 1.8.

12. Margins of median groove of pronotum: 0 = more or less parallel along whole length; 1 = maximum width near midlength; 2 = maximum width anteriad to midlength.

13. Thickness of margins of median groove of pronotum: 0 = narrow; 1 = evenly thickened along entire length; 2 = distinctly more strongly thickened in proximal part than in distal part.

14. Setae on odd intervals of elytra: 0 = recumbent; 1 = erect.

15. Setae on declivity of elytra: 0 = not denser and not more strongly erect than on other parts of elytra; 1 = clearly denser and more strongly erect than on other parts of elytra (Fig. 25).

16. Setae on tibiae: 0 = external setae much wider than internal ones (Fig. 26); 1 = all setae of the same width (Fig. 27).

17. Shape of setae on elytral intervals: 0 = ovoid; 1 = elliptical.

18. Length of setae on elytral intervals: 0 = shorter than one interval width; 1 = as long as or longer than one interval width.

19. Scales on body: 0 = oval; 1 = slender (hair-like).

20. Setae on sides of pronotum: 0 = recumbent; 1 = erect.

21. Setae on femora: 0 = elliptical or slender (hair-like); 1 = ovoid.

22. Lobes of third tarsomere: 0 = lobes very small (Fig. 17); 1 = lobes distinct, long (3rd tarsomere longer than wide); 2 = lobes distinct, very broad (Fig. 19).

23. Elytra: 0 = ovoid, much wider than pronotum; 1 = ovoid, slightly wider than pronotum; 2 = ovoid-elliptical; 3 = elongate-elliptical.

24. Female sternite VIII, ratio between total length and length of spiculum ventrale: 0 = < 2; 1 = > 2.

25. Female sternite VIII, ratio length/width of plate: 0 = > 2; 1 = < 2.

26. Female sternite VIII, apex of plate: 0 = convex; 1 = not convex.

27. Female sternite VIII, width of proximal part of plate: 0 = narrower or as wide as distal half; 1 = wider than distal half.

28. Female sternite VIII, sides of plate: 0 = evenly divergent posteriad along the entire length; 1 = strongly divergent posteriad in distal part and sub-parallel in proximal part.

29. Length of apical lamella of penis: 0 = short; 1 = long, C-shaped in profile.

30. Truncation of apical lamella of penis: 0 = truncated; 1 = rounded.

31. Width of apical lamella of penis: 0 = not very slender; 1 = very slender.

32. Penis thickness at base compared to remainder of penis: 0 = not thickened; 1 = thickened.
33. Curvature of penis: 0 = evenly curved along entire length; 1 = more strongly curved in proximal than in distal part.
34. Length of internal sac: 0 = long, genital sclerite placed in correspondence of apex of temones; 1 = moderately long, sclerite placed in correspondence of midlength of temones; 2 = short, sclerite placed at base of temones.
35. Ratio between length and width of tectum of genital sclerite (sclerite: Figs. 28–53): 0 = very long, ratio length/width > 4; 1 = long, ratio length/width 3–4; 2 = ratio l/w 2–3; 3 = ratio l/w 1.5–2; 4 = subquadrate, ratio l/w 1.1–1.3.
36. Shape of tectum of genital sclerite: 0 = parallel-sided; 1 = distinctly broadened basad; 2 = slightly broadened apicad.
37. Apex of tectum of genital sclerite: 0 = with a downward curved plate; 1 = lacking any specialized structure, broadly leaf-shaped bifurcate (Fig. 28); 2 = lacking any specialized structure, broadly expanded laterally (MEREGALLI 1987: fig. 250); 3 = lacking any specialized structure, nearly truncated (Fig. 34); 4 = with a long median horn, only divided at apex (Figs. 29, 30); 5 = with a short, barely developed and apically undivided horn (Fig. 32); 6 = with a moderately prominent, long divided horn (Fig. 39); 7 = with a strongly prominent long divided horn, extended much beyond anterior margin of tectum (Figs. 48, 51).
38. Anterior arms of genital sclerite: 0 = absent; 1 = very minute at side of apical plate; 2 = short, thick, with nearly the same thickness from base to apex, sharply curved downwards, only base of arm visible from above (Fig. 36: dorsal view; Fig. 40: lateral view); 3 = short (much shorter than apical horn), thick at base and narrowed apicad, not or barely curved downwards, entire arm visible from above (Fig. 29); 4 = moderately elongated, thick at base and slightly narrowed apicad, distinctly and evenly curved downwards, the proximal half of its length visible from above (Fig. 32); 5 = very long, narrow, sharply curved in its proximal part, reaching underside of sclerite (Fig. 46); 6 = very long and narrow, prominent forwards beyond margin of tectum, not tapered apicad (Fig. 51); 7 = same as 6 but distinctly tapered apicad (Fig. 48).
39. Position of base of valves of genital sclerite: 0 = at midlength of sclerite; 1 = at base of sclerite; 2 = valves joined basad behind base of sclerite.
40. Distal extension of valves of genital sclerite: 0 = reaching apex of tectum; 1 = not reaching apex of tectum (Figs. 48–53); 2 = surpassing apex of tectum.
41. Lower margin of valves of genital sclerite: 0 = entire (Fig. 46); 1 = bilobate (Fig. 37).
42. Structure of apical part of valves of genital sclerite: 0 = without an apical lobe or a subapical process; 1 = with an apical lobe; 2 = with a subapical process.
43. Valves of genital sclerite in lateral view: 0 = scarcely thickened; 1 = thick.
44. Setae on lower part of genital sclerite: 0 = absent; 1 = scarce; 2 = dense (and prominent beyond anterior margin of sclerite).
45. Food-plants: 0 = mosses; 1 = *Saxifraga*.

2.3. Molecular data

Total DNA was extracted by grinding the whole animal body in 400 µl of 5M guanidine-isothiocyanate; removal of proteins was accomplished by adding 400 µl of 4M sodium perchlorate and chloroform extraction. Total DNA was precipitated from the upper phase by isopropylic alcohol precipitation and resuspended in TE buffer. A 829 bp fragment of mitochondrial cytochrome oxidase subunit I (COI) was amplified with the following primers (HUGHES & VOGLER 2004): forward C1-J-2183 (Jerry: 5'-CAACATTTATTTTGATTTTTTGG-3') and reverse L2-N-3014 (Pat: 5'-TCCAATGCACTAATCTGCCATATTA-3'). All PCRs were performed in a volume of 20 µl with HotStarTaq Master Mix (Qiagen). 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 aligned with GeneStudioPro (http://www.genestudio.com) using default parameters and correcting ambiguities manually. Sequences were trimmed at the ends reducing the length to 775 bp. Multiple sequence alignment was performed with ClustalX 2.0.10 (LARKIN et al. 2007) using default parameters. Some populations were, in the first round, represented only by evident nuclear pseudogenes (Numts of COI) without an open reading frame and with a big gap in the middle of the sequence (about 30 bp). To obtain mitochondrial COI sequences from these populations we made a second round using a pair of primers that corresponded with the gap: forward PGKf 5'-CATCTATCGACATTATCCTTC-3', reverse PGKr 5'-GAAGGATAATGTCGATAGATG-3'. These primers are reciprocally complementary and we used the former in combination with Pat, the latter in combination with Jerry. We used the reaction profile described above except for the extension time, which was reduced to 1 min. All the sequences used for the phylogenetic analysis that were different not only because of missing data were deposited in GenBank (see Table 3 for accession numbers).

From 36 specimens (listed in Table 3) we obtained 39 sequences, i.e. some specimens yielded different se-

Table 3. Specimens used in molecular analysis. In light-grey the primers that amplified Numts without an open reading frame, in medium-grey the primers that amplified sequences with an open reading frame but considered to be Numts (see text). Identical accession number for different specimens mean that they share the same haplotype. Sequences that differed only because of missing data were not submitted to GenBank, but were used in the phylogenetic analysis; they are indicated with "ns".

Species	Locality	Couple(s) of primers	GenBank accession number
D. doderoi	Colle di Fremamorta 2600 m	Jerry+Pat	[Numt only]
		Jerry+Pat	[Numt only]
		Jerry+Pat	[Numt only]
		Jerry+Pat	[Numt only]
	Colle del Mulo 2500 m	Jerry+Pat	[Numt only]
D. margaritae	Colle della Vecchia 2500/2600 m	PGKf+Pat	KC858990
		Jerry+Pat & PGKf+Pat	≙ KC858990 (ns)
		PGKf+Pat	KC858991
		Jerry+PGKr & PGKf+Pat	[Numt only]
D. sulcipennis	Colle del Nivolet 2800 m	Jerry+Pat	KC858988
		Jerry+Pat	KC858989
		Jerry+Pat	≙ KC858989 (ns)
		Jerry+Pat & PGKf+Pat	[Numt only]
		Jerry+Pat & PGKf+Pat	[Numt only]
D. manueli	Monte Rocciamelone 2900 m	Jerry+Pat	KC858978
		Jerry+Pat	KC858979
D. bischoffi	Colle Bardoney 2900 m	Jerry+Pat	KC858980
		Jerry+Pat	KC858980
		Jerry+Pat	KC858980
		Jerry+Pat	KC858980
		Jerry+Pat	KC858980
		Jerry+Pat	KC858980
		Jerry+Pat	KC858980
D. baudii	Monte Maniglia 2900 m (02)	Jerry+Pat	KC858985
		Jerry+Pat	KC858985
		Jerry+Pat	KC858985
	Colle della Vecchia 2600 m (01)	Jerry+Pat	KC858981
D. stierlini	Colle Bardoney 2800 m	Jerry+Pat	KC858982
		Jerry+Pat	KC858982
		Jerry+Pat	KC858982
		Jerry+Pat	≙ KC858982 (ns1)
		Jerry+Pat	≙ KC858982 (ns2)
D. maculosus	Gornergrat (CH) 2600 m (01)	Jerry+Pat & PGKf+Pat	KC858983
	Colle di Fremamorta 2600 m (02)	Jerry+Pat	KC858984
	Colle della Vecchia 2500 m (03)	Jerry+Pat	KC858986
	Colle dell'Agnello 2800 m (04)	Jerry+Pat	KC858987

quences. With this original set of COI(-like) sequences we proceeded in the following way:

(1) Some of the sequences from different but conspecific specimens were identical and were computed only once in the alignments, thus arriving at 31 haplotypes.

(2) Nuclear copies of mitochondrial genes (Numts, a class of pseudogenes) are commonly found in eukaryotic organisms. Two ways to recognize them are the presence of highly unexpected nodes in the tree topologies (Bensasson et al. 2001) and inconsistencies in the reading frame for association with amino acids (including occurrence of stop codons). We thus examined our dataset for Numts by inspecting the phylogenetic trees and by looking for the presence of an open reading frame (orf). For the former purpose we built a neighbor-joining tree for the 31 haplotypes (not shown) with MEGA 5.04 (Tamura et al. 2011), support was computed with 100 bootstrap replications. Three haplotypes without an orf clustered together with maximum support (bootstrap value = 100%). This haplotype clade was excluded from successive analysis. All other haplotypes had an open reading frame. In addition, all other clades of the neighbor-joining tree included haplotypes that came from specimens of the same population or of the same species, except for one clade. This last clade contains 10 nearly identical haplotypes (maximum pairwise distance = 2%) from 4 very differentiated species (*D. doderoi*, *D. margaritae*, *D. sulcipennnis* and *D. maculosus*) and has a maximum bootstrap value (100%). Among these species, *D. margaritae*, *D. sulcipennnis* and *D. maculosus* yielded other, more diverse haplotypes in addition, but *D. doderoi* did not. The presence of nearly identical haplotypes from very different species (*D. do-*

deroi, *D. margaritae / sulcipennis*, and in particular *D. maculosus*) in the same clade, the observation that different primers on the same template gave different COI-like haplotypes and the fact that these occurrences are limited to only one haplotype clade suggest that this clade represents only Numts, even if they have an open reading frame. We excluded these haplotypes according to their abnormal highly conservative behaviour (for which we have no explanation), and especially due to the additional occurrence in the same specimens of other COI sequences showing normal rates of divergence. We consider the latter the genuine transcribed mtCOI genes and included only these in our analysis.

(3) After exclusion of putative Numts, the alignment included 18 different haplotypes from 5 large-sized and 2 small-sized species of *Dichotrachelus* (data in Table 3; alignment in Electronic Supplement), all 775 bp long, except those belonging to *D. margaritae*, which are about 400 bp (towards the 3' extremity). The final alignment does not contain sequences from *D. doderoi*, from which we only obtained Numts.

In a first analysis three haplotypes from the Cyclominae outgroup taxa were added. In a further analysis, the sequences of 159 further species of Curculionoidea retrieved from GenBank were added to the sequence of the first analysis.

2.4. Phylogenetic analysis: morphology

Maximum Parsimony analysis (MP) was performed with TNT 1.1 (GOLOBOFF et al. 2008). 10000 replications of stepwise addition with random addition sequence (ras) of taxa and tree-bisection-reconnection (tbr) branch swapping, saving up to 10000 trees for replication (no replication reached this number), were used to find the best trees. Group support was computed with 10000 bootstrap replications.

The maximum likelihood (ML) tree was inferred with raxmlGUI1.1 (SILVESTRO & MICHALAK 2011) using a MarkovK + Γ model. Support values were computed with 1000 bootstrap replications.

Bayesian inference (BI) was performed using MrBayes 3.2 (RONQUIST et al. 2012). We ran two runs with 4 MCMC chains, each for 10 million generations under a binary MarkovK + Γ model, sampling every 1000 generations. The first 25% generations were discarded (burn-in) and convergence was evaluated with the average standard deviation of split frequencies (0.006). Goodness of mixing was assessed looking at the acceptance rate of swaps between adjacent chains, following RONQUIST et al. (2009).

To infer the geographical range of ancestral species we used the Statistical Dispersal-Vicariance Analysis (S-DIVA) method (YU et al. 2010) implemented in RASP 2.0 beta (YU et al. 2013). Trees sampled by MCMC of morphological analysis were used discarding the first 25% as burn-in. One or two different areals were assigned to actual species choosing between six: Spain and North Africa (**O**), Pyrenees (**D**), western and south-western Alps (south of the Moncenisio Pass) (**A**), north-western Alps (between the Moncenisio Pass and Mt. Rosa) (**B**), Central Alps (from Lake Maggiore to Lake Garda) (**E**), and Eastern Alps (from Lake Garda to the Slovenian Alps) (**C**).

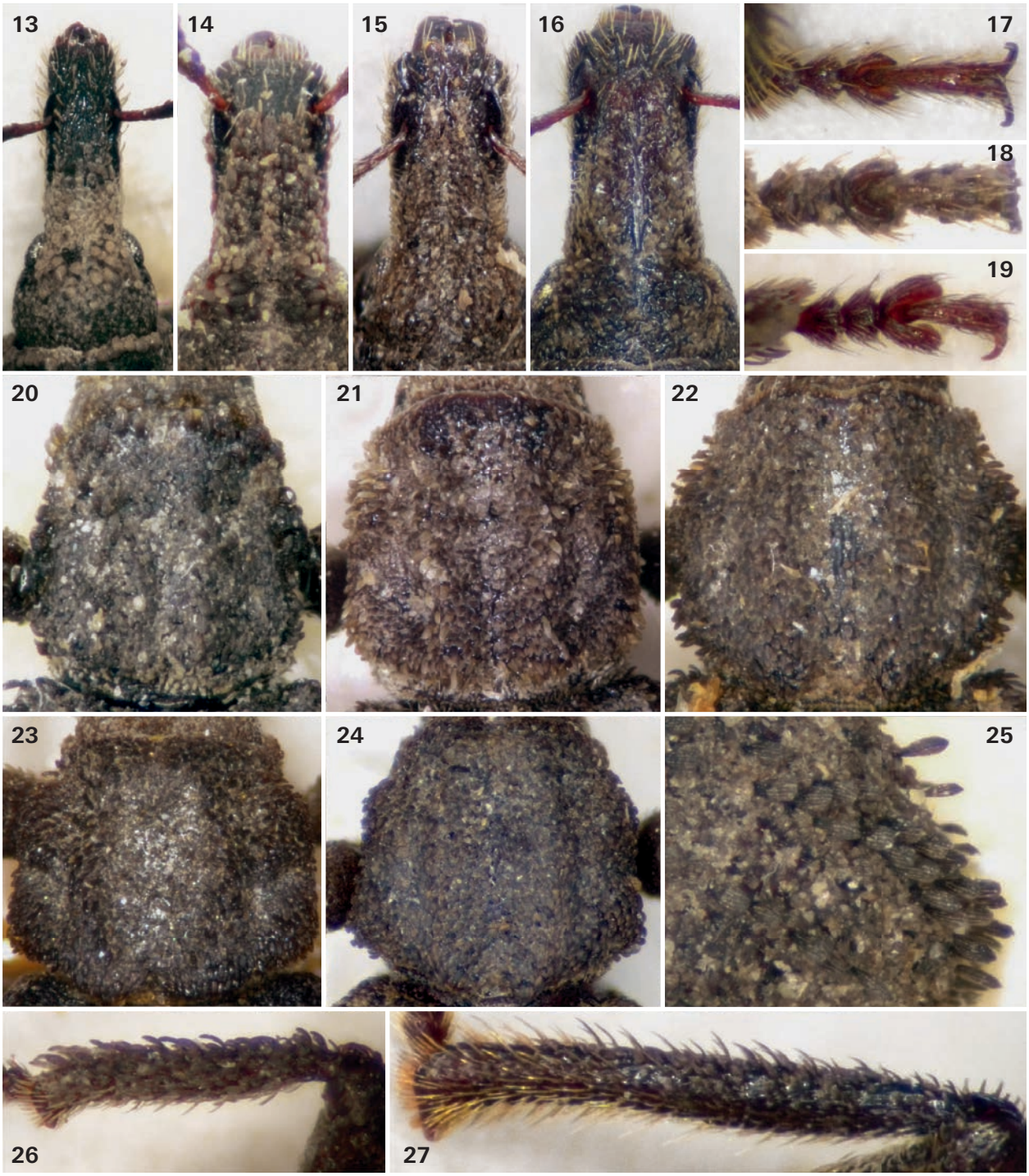
2.5. Phylogenetic analysis: COI

Bayesian Inference (BI) was estimated on the nucleotide sequences of the 18 *Dichotrachelus* haplotypes and the 3 Cyclominae outgroup sequences, using MrBayes 3.2 (RONQUIST et al. 2012). We ran two runs with 4 chains, each for 2 million generations, sampling every 500 generations. The chains were let 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 modeled with a 4 categories discretized Γ distribution and with a proportion of invariable sites. The first 25% generations were discarded (burn-in) and convergence was evaluated with the average standard deviation of split frequencies (0.002). Goodness of mixing was assessed looking at the acceptance rate of swaps between adjacent chains, following RONQUIST et al. (2009).

Maximum Likelihood (ML) inference was estimated on the nucleotide sequences of the 18 *Dichotrachelus* haplotypes and the 3 Cyclominae outgroup sequences. jModelTest 0.1.1 (POSADA 2008) was used to find the best models among 88 belonging to the GTR family, with the corrected Akaike information criterion (AICc) that chose the GTR + I + Γ. ML was performed under this model using MEGA 5.04 (TAMURA et al. 2011). Γ distribution was approximated with 4 categories and only sites with more than 80% of unambiguous nucleotides were included in the analysis. Support values were computed with 500 bootstrap replications.

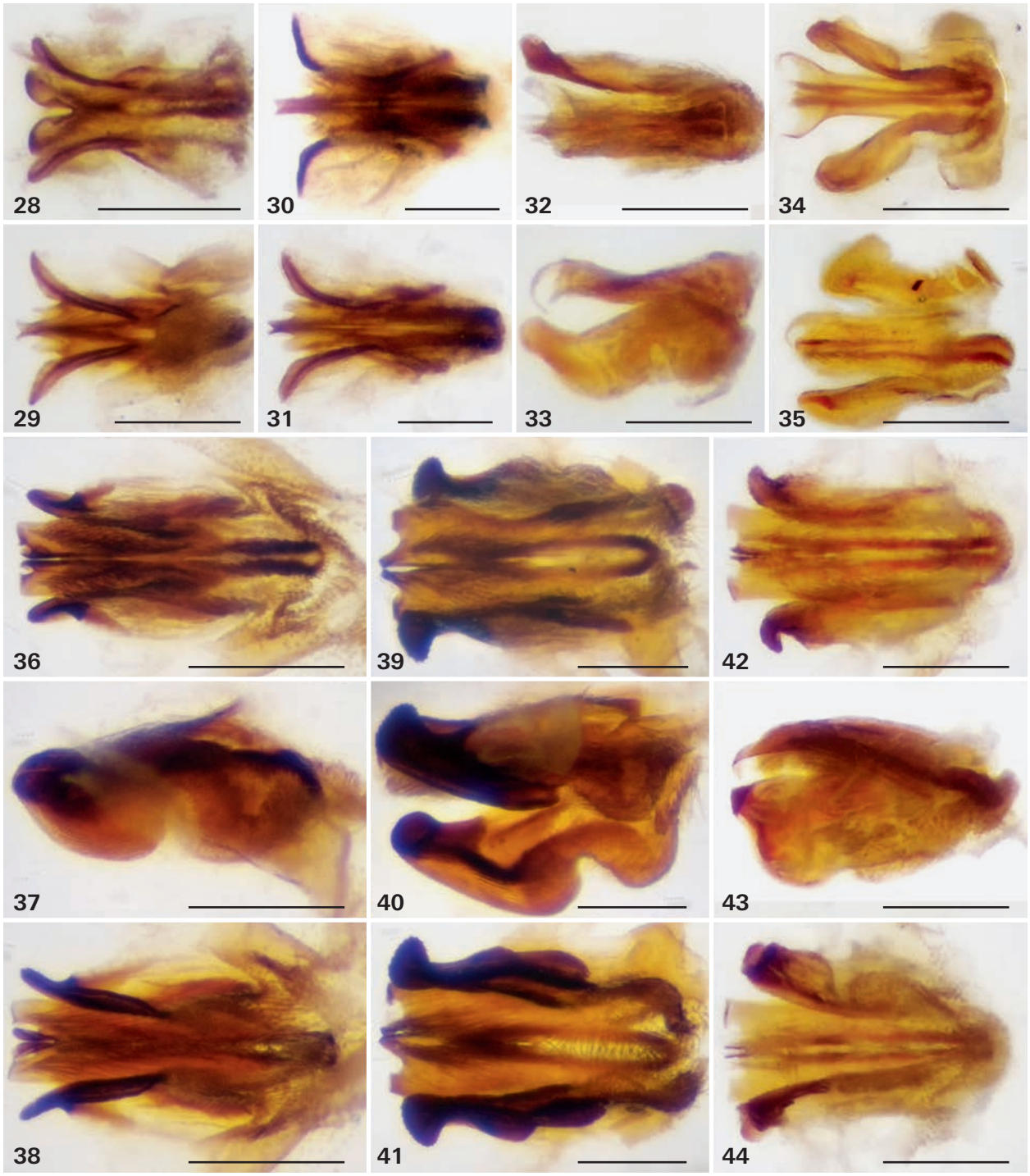
BI (but not ML) was additionally estimated on the amino acid sequences (aas) of the sample included in the previous analyses plus 159 taxa of Curculionoidea, using MrBayes 3.2. The aas were downloaded from NCBI (the nucleotide sequences we had amplified were translated into aas with Mega 5.04). We ran two runs with 4 chains under a mixed amino acid model (HUELSENBECK et al. 2004), each for 1 million generations, sampling every 500 generations.

The ages of phylogenetic splitting events were estimated on the *Dichotrachelus* and Cyclominae nucleotide sequences, and on the *Dichotrachelus*-only nucleotide sequences. Three different molecular clock models were tested with Bayes factor (B_{10}): no-clock model, strict clock model, and uncorrelated gamma rate model (LEPAGE et al. 2007). MrBayes 3.2 was used to compute the marginal likelihood of the three models with the step-



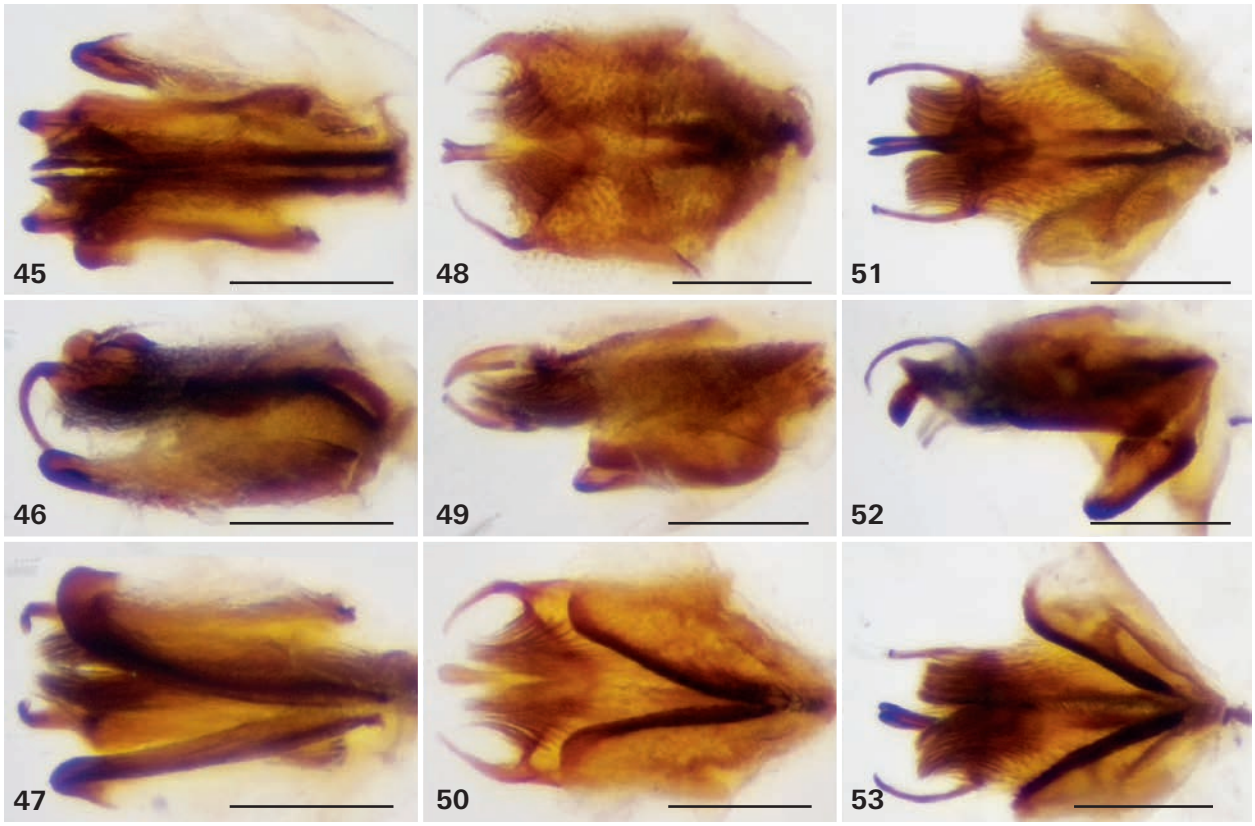
Figs. 13–27. *Dichotrachelus*, parts of the body. Rostrum of: **13**: *D. berberus*, Djbel Tazzeka. **14**: *D. stierlini*, Colle dell'Arietta. **15**: *D. margaritae*, Cima Ciantiplagna. **16**: *D. manueli*, Rocciamelone. Fore tarsus of: **17**: *D. manueli*, Rocciamelone. **18**: *D. margaritae*, Cima Ciantiplagna. **19**: *D. rudeni*, Col de Balme. Pronotum of: **20**: *D. rudeni*, Col de Balme. **21**: *D. manueli*, Rocciamelone. **22**: *D. margaritae*, Cima Ciantiplagna. **23**: *D. sulcipennis pedemontanus*, Pian della Mussa. **24**: *D. sulcipennis bernhardinus*, Col Ferret. **25**: Apical part of elytral suture of *D. rudeni*, Col de Balme. Fore tibia of: **26**: *D. rudeni*, Col de Balme. **27**: *D. manueli*, Rocciamelone.

ping stone algorithm (Xie et al. 2012): a 50 steps 2.5 million generations analysis sampling every 500 generations was performed using 2 runs with 4 chains, each under the GTR model. Heterogeneity of substitution rates among different sites was modeled with a 4 categories discretized Γ distribution and a proportion of invariable sites. The no-clock model was strongly preferred: $2\ln B_{10}$ = 54 against the strict clock model and 50 against the uncorrelated gamma rate model (Ln marginal likelihood = –4058; –4031; –4033 for the no-clock, strict clock, and uncorrelated gamma rate model, respectively). The same test was repeated without the three Cyclominae outgroup species and with slightly changed parameters: a 50 steps 3 million generations analysis sampling every 500 gen-



Figs. 28–44. *Dichotrachelus*, male genital sclerite. **28**: *D. stierlini*, Colle dell'Arietta, ventral. **29**: *D. rudeni*, Col de Balme, ventral. **30**: *D. imhoffi*, Val Seriana, dorsal. **31**: *D. imhoffi*, Val Seriana, ventral. **32**: *D. luzei*, Mt. Grintovec, dorsal. **33**: *D. luzei*, Mt. Grintovec, lateral. **34**: *D. kahleni*, Mt. Sernio (paratypus), dorsal. **35**: *D. philippi*, Mt. Pasubio (paratypus), dorsal. **36**: *D. grignensis* cf., Pizzo Arera, dorsal. **37**: *D. grignensis* cf., Pizzo Arera, lateral. **38**: *D. grignensis* cf., Pizzo Arera, ventral. **39**: *D. baudii*, Cima Ciantiplagna, dorsal. **40**: *D. baudii*, Cima Ciantiplagna, lateral. **41**: *D. baudii*, Cima Ciantiplagna, ventral. **42**: *D. bischoffi*, Val Clavalité, dorsal. **43**: *D. bischoffi*, Val Clavalité, lateral. **44**: *D. bischoffi*, Val Clavalité, ventral. Bar: 250 µm.

erations was performed using 2 runs with 4 chains each. Chains were let free of change model of the GTR family using reversible jump MCMC (HUELSENBECK et al. 2004). Heterogeneity of substitution rates among different sites was modeled with a 4 categories discretized Γ distribution and a proportion of invariable sites. To help convergence a topological constraint was imposed: all the *Saxifraga*-associated species were forced to form a monophyletic group. With this new dataset Bayes factor strongly prefers the uncorrelated gamma rate model against both the no-clock model ($2\ln B_{10}$ = 520 with Ln marginal likelihood of the uncorrelated gamma



Figs. 45–53. *Dichotrachelus*, male genital sclerite. **45**: *D. manueli*, Mt. Rocciamelone, dorsal. **46**: *D. manueli*, Mt. Rocciamelone, lateral. **47**: *D. manueli*, Mt. Rocciamelone, ventral. **48**: *D. sulcipennis bernhardinus*, Col Ferret, dorsal. **49**: *D. sulcipennis bernhardinus*, Col Ferret, lateral. **50**: *D. sulcipennis bernhardinus*, Col Ferret, ventral. **51**: *D. linderi*, Mt. Canigou, dorsal. **52**: *D. linderi*, Mt. Canigou, lateral. **53**: *D. linderi*, Mt. Canigou, ventral. Bar: 250 μm.

rate model = –3323 and Ln marginal likelihood of the no-clock model = –3060) and the strict clock model ($2\ln B_{10}$ = 590 with Ln marginal likelihood of the strict clock model = –3028). According to Kass & Raftery (1995) this is very strong evidence in favour of the uncorrelated gamma rate model, which we eventually used for dating.

Phylogenetic trees were calibrated with a sequence divergence rate of 2.1% per My according to Papadopoulou et al. (2010) under the uncorrelated gamma rate model (Lepage et al. 2007). Uncertainty of the substitution rate is evaluated in Bayesian inference by changing the rate with a proposal mechanism at every generation. A Bayesian analysis with the same parameters (except the model and the parameters related to the clock) of the no-clock analysis was performed. To help convergence a topological constraint was imposed: all *Saxifraga*-associated species were forced to form a monophyletic group. Prior on the base substitution rate was set normal distributed with mean 0.021 and variance 0.005; prior on tree age was set exponential distributed with mean 1/0.021. The first 25% generations were discarded (burn-in) and convergence was evaluated with the average standard deviation of split frequencies (0.004). Goodness of mixing was assessed looking at the acceptance rate of swaps between adjacent chains, following Ronquist et al. (2009).

3. Results

3.1. Phylogenetic inference

Resulting trees are shown in Figs. 54, 55. In the morphological analysis, parsimony with equal weights resulted in 4 MP trees (tree length = 110, CI = 0.75, RI = 0.89), and a majority rule consensus tree was generated. The topologies found with ML and BI (Fig. 54) were relatively congruent with the topology found with MP, particularly for the clades with considerable statistical support in MP.

The three included small-sized alpine species and the *Saxifraga*-associated ones clustered independently from the Spanish and Moroccan small-sized *Dichotrachelus* (but note that the Moroccan *D. berberus* was defined as the farthest outgroup taxon, see section 2.1.). Two of the alpine small-sized species, *D. stierlini* and *D. maculosus*, together formed the sister to the remaining taxa in BI and MP, but they formed a monophyletic clade together with *D. rudeni* and *D. imhoffi* in ML (although not supported*). *Dichotrachelus rudeni* and *D. imhoffi*

* We use "not supported" if support value is < 50%, "moderately supported" if 51–90%, "strongly supported" if > 90%.

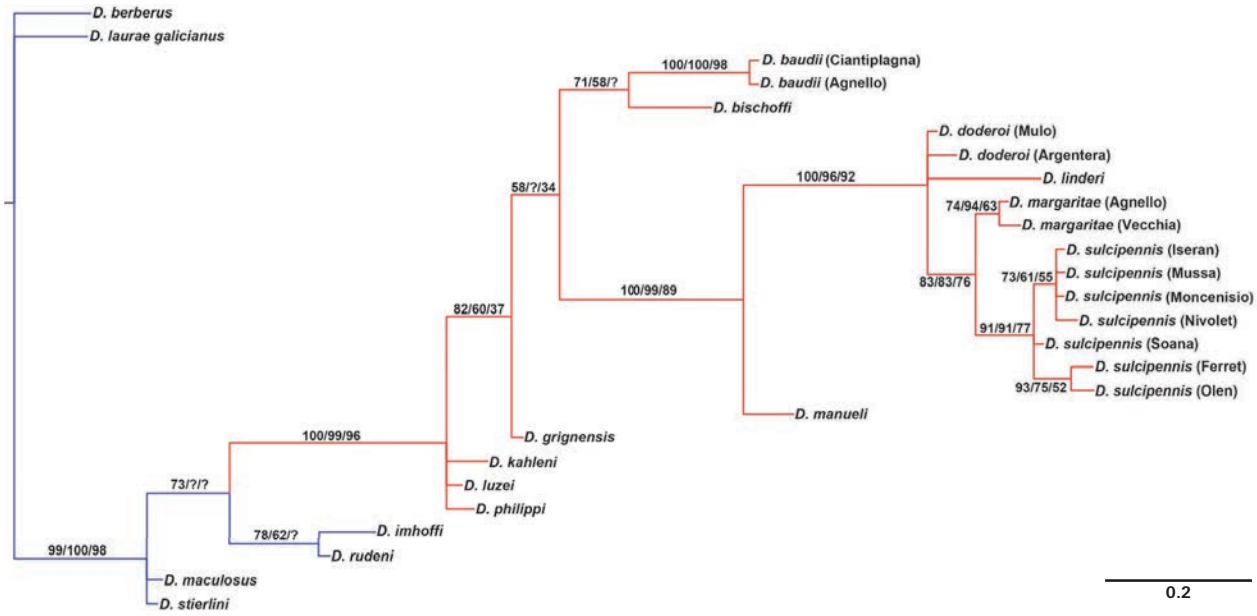


Fig. 54. Morphological analysis: Bayesian consensus tree (majority rule 50%). Support values are reported in percentages and are in the following order: Posterior probability (BI) / Bootstrap value (ML) / Bootstrap value (MP). '?' means that the group was not present on the ML tree or in the MP trees. For graphical reason support values are not reported for all clades. The red sub-tree highlights the *Saxifraga*-associated monophyletic group of species. The complex of "large-sized" species is congruent with this but additionally includes the moss-feeding *D. imhoffi*. See Table 1 for details on populations.

resulted as sister species in BI and ML, with moderate support (respectively, 78% posterior probability [= pp] and 62% bootstrap [= bs]). All these species feed on mosses, but whereas *D. imhoffi* is a "large-sized" species, the remaining ones including *D. rudeni* are "small-sized" ones.

The *Saxifraga*-associated species formed a strongly supported clade in all analyses. Those native to the eastern Alps (east of Lake Garda, see section 2.4.) (*D. luzei*, *D. kahleni*, *D. philippi*) were in a polytomy that additionally gives origin to a moderately supported clade comprising all central- (between Lake Maggiore and Lake Garda) and western- (west of Lake Maggiore) alpine taxa. The single central- (*D. grignensis*) and two of the western alpine taxa (*D. bischoffi* and *D. baudii*) together formed a clade in ML (not supported: 47% bs), with *D. grignensis* sister to a moderately supported *D. bischoffi* + *D. baudii* clade (58% bs). In MP and BI *D. grignensis* was sister to all western alpine species. The *D. bischoffi* + *D. baudii* clade was supported by 71% pp in BI. In the topology resulting from the molecular analyses *D. baudii* originated from a further basal dichotomy, from among the paraphyletic small-sized *Dichotrachelus* (Fig. 55), with an almost full support in BI (98% pp), but barely supported in ML (54% bs).

The four other species from the western Alps (*D. manueli*, *D. doderoi*, *D. sulcipennis*, *D. margaritae*) plus *D. linderi* from the Pyrenees formed a clade fully supported in the morphological analysis, and strongly supported in the molecular analysis (only *D. manueli*, *D. sulcipennis*, and *D. margaritae* included). *Dichotrachelus manueli* was sister to the four other species, again with a full or almost full support. The only uncertainty regarded a polytomy comprising two populations of *D. doderoi* (referable to different subspecies) from the western Alps, *D. linderi* from the Pyrenees, and *D. margaritae* + *D. sulcipennis*, for which no resolution could be retrieved with any support. The differentiation of *D. margaritae* and *D. sulcipennis* at specific level was supported by all methods. The taxonomic fragmentation of *D. sulcipennis* was confirmed by our study, even though the single groups did not always correspond to the currently defined subspecies.

The BI estimated independently on the 21 genital vs. the 23 non-genital characters (food-plant character 45 was excluded) gave congruent results. The support to several nodes was lower, but both topologies were overall identical with the topology resulting from the complete matrix for the major clades, occasionally with some changes in the sequence of the species in comb-like parts of the tree. The parsimony based ILD test (FARRIS et al. 1995) did not reject the null hypothesis of congruence between the genital and non-genital datasets (p = 0.5, 1000 random partitions). The test was performed with TNT 1.0 (GOLOBOFF et al. 2008), using a script written by Mark Siddal available at http://tnt.insectmuseum.org/index.php/Scripts/ild.run (accessed May 29, 2013).

In the BI estimated on the COI-derived amino acid sequences of 169 species of Curculionoidea (159 species retrieved from GenBank, plus the 7 species of *Dichotrachelus* and the 3 species of Cyclominae used in our study), the genus *Dichotrachelus* clustered in an independent, almost fully supported clade (98% posterior probability), only distantly associated with the other spe-

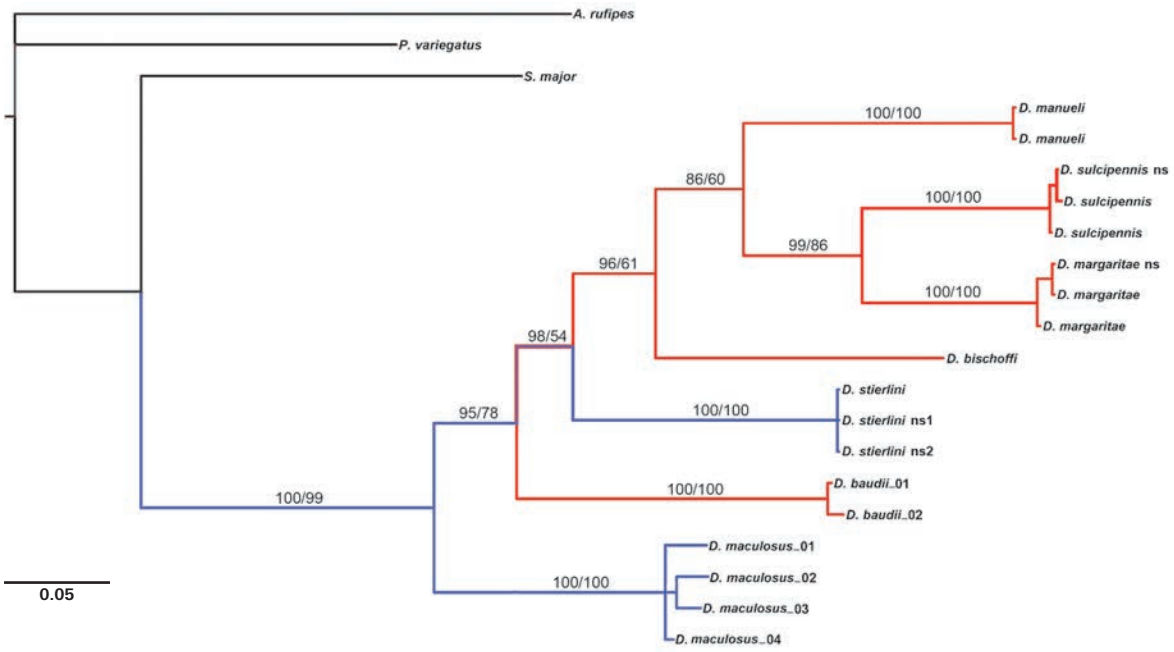


Fig. 55. Molecular analysis on mtCOI: Bayesian consensus tree (majority rule 50%). Support values are reported as percentages and are in the following order: posterior probability (BI) / bootstrap value (ML). See Table 3 for details on specimens; conspecific terminals not numbered are always from the same locality. Scale bar unit: expected substitution per site. Support values for nodes inside groups constituted of conspecific specimens are not reported. For "ns" see legend Table 3.

cies of Cyclominae, the nearest one being *Aoplocnemis rufipes*, which was obtained as the sister of *Dichotrachelus* (not supported: 43% pp). The Cyclominae clade is part of a strongly supported (94% pp) clade encompassing taxa belonging to the Cyclominae, Entiminae, Gonipterini and Hyperini. Within *Dichotrachelus*, this analysis yielded the same relationships as obtained with only the 3 Cyclominae outgroup taxa (as in Fig. 55).

3.2. Genetic distance

The mean genetic distance (*p*-distance) between *Dichotrachelus* species was 14.6% (range 11.0%–16.8%). The mean *p*-distance between the two genetically most similar species, *D. sulcipennis* and *D. margaritae*, was 11%, much higher than the threshold of 2% for species delimitation proposed by Hebert et al. (2003). Few data are available for inter- and intraspecific divergence in weevils, but such a threshold is probably too low, at least in the case of taxa distributed in isolate relict populations, which may have intraspecific divergence higher than 2%. Among about 50 species of *Trigonopterus* Fauvel, 1862 (Curculionidae) from a mountain range in New Guinea, Riedel et al. (2009) found an interspecific mean *p*-distance of 20.5%, with a minimum distance of 16.5%; intraspecific distance ranged from 0.0% to 8.8% (mean 1.2%). The genetic distance between *D. sulcipennis* and *D. margaritae* has thus an intermediate value between the minimum interspecific and the maximum intraspecific distances in *Trigonopterus*. The intraspecific distance between different populations of the same species of *Dichotrachelus* (*D. baudii* and *D. maculosus*) ranged from 0.2% to 2.7% (mean 1.6%), thus our data for *D. sulcipennis* and *D. margaritae* support specific differentiation. The maximum intrapopulation divergence values were 0.4% for *D. sulcipennis* and 0.5% for *D. margaritae*.

3.3. Molecular clock

Fig. 56

Node ages were calculated with a 2.1% My^{-1} substitution rate, an estimation based on 13 studies of mtDNA divergence rates in insects (Papadopoulou et al. 2010). Criticism of molecular clocks has often been expressed, in particular variation among taxa and intrinsic theoretical limits of the procedure have been discussed (Bromham & Penny 2003; Schwartz & Maresca 2006; Gibbons 2012; etc.). The need to calibrate the clock with the known absolute age of evolutionarily independent events was also emphasized (Ho 2008). In *Dichotrachelus* generation time should also be considered, since these weevils have only a few months a year of active life, due to the extremely harsh climate in their high altitude alpine habitats. The life cycle seems to be at least two years long (Meregalli 1980), and it is known that generation time affects the nucleotide substitution rate of

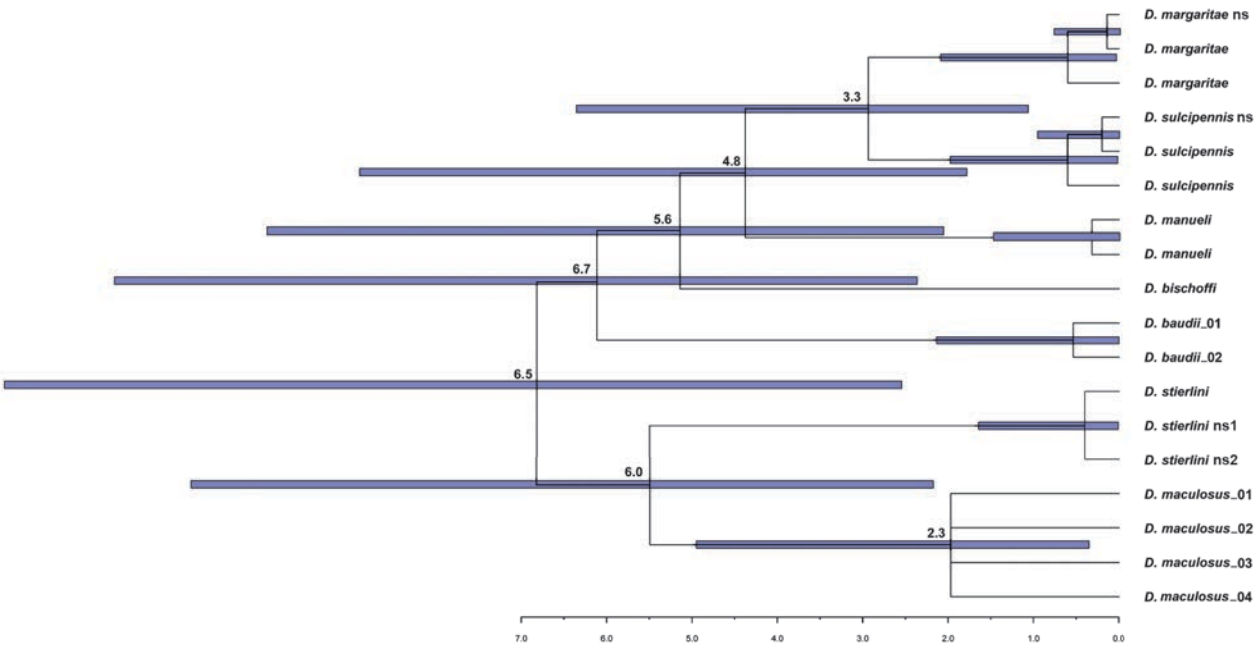


Fig. 56. Molecular clock. Bayesian consensus tree from molecular analysis (majority rule 50%) with substitution rate 2.1% per My. Node labels indicate the mean of node age estimations, only nodes older than 2.0 My are labeled. 95% of estimation fall in the blue bars. Scale unit are My. This analysis was performed on the dataset that included only the *Dichotrachelus* haplotypes, according to the results of the molecular clock testing (see Material and Methods). See Table 3 for details on the specimens and for "ns".

molecular evolution in invertebrates: species with lower generation turnover tend to have lower rates (THOMAS et al. 2010). Mean age estimation of divergence between the *Saxifraga*-associated lineage and its moss-associated sister taxon was about 7.5 My, and divergence between the genetically most similar *D. sulcipennis* and *D. margaritae* was about 3.3 Mya. These ages can be expanded to about 14–3.5 My resp. 7.0–1.4 My with a 95% confidence interval.

3.4. Geographical range of ancestors

Fig. 57

When the first radiation of the genus *Dichotrachelus* occurred, in late Oligocene or early Miocene, as indicated by MEREGALLI (1987), the land masses that later split and gave origin to the present-day orography and geography were still united within a single plate at the north-western margin of the Tethys ocean (POPOV et al. 2004). We only included a few of the small-sized species, and without knowledge of phylogeny of the entire genus nothing can be said about the geographic range of its ancestor. For the alpine species (including both small- and large-sized ones) plus *D. linderi* from the Pyrenees, and also for the *Saxifraga*-associated clade, Statistical Dispersal-Vicariance Analysis (S-DIVA) suggested with full support that their last common ancestor (LCA) was located in the Alps. Within the *Saxifraga*-associated clade, as expected from the present-day distribution, the complex of *D. luzei*, *D. philippi* and *D. kahleni* derived from a radiation in the central to eastern Alps. For the western alpine species plus *D. linderi* (*D. baudii*, *D. bischoffi*, *D. manueli*, and the clade encompassing the four other species) results indicate an LCA with a western-northwestern alpine range.

4. Discussion

4.1. *Dichotrachelus* relationships

The analysis of the phylogenetic relationships of *Dichotrachelus* with the Cyclominae and other subfamilies and families of Curculionoidea indicates that the genus belongs in the Cyclominae + Entiminae + Gonipterini + Hyperini clade. Within this clade, its position is very isolated, and even its belonging to the Cyclominae may be questionable. This confirms the results of the morphological study of OBERPRIELER (2010). We report these data since they add previously unavailable information, but it should be stressed that this analysis is very preliminary and anyway was not within the scope of the present study. More genes, including nuclear genes, should be used to infer with sounder evidence the relationships of *Dichotrachelus*.

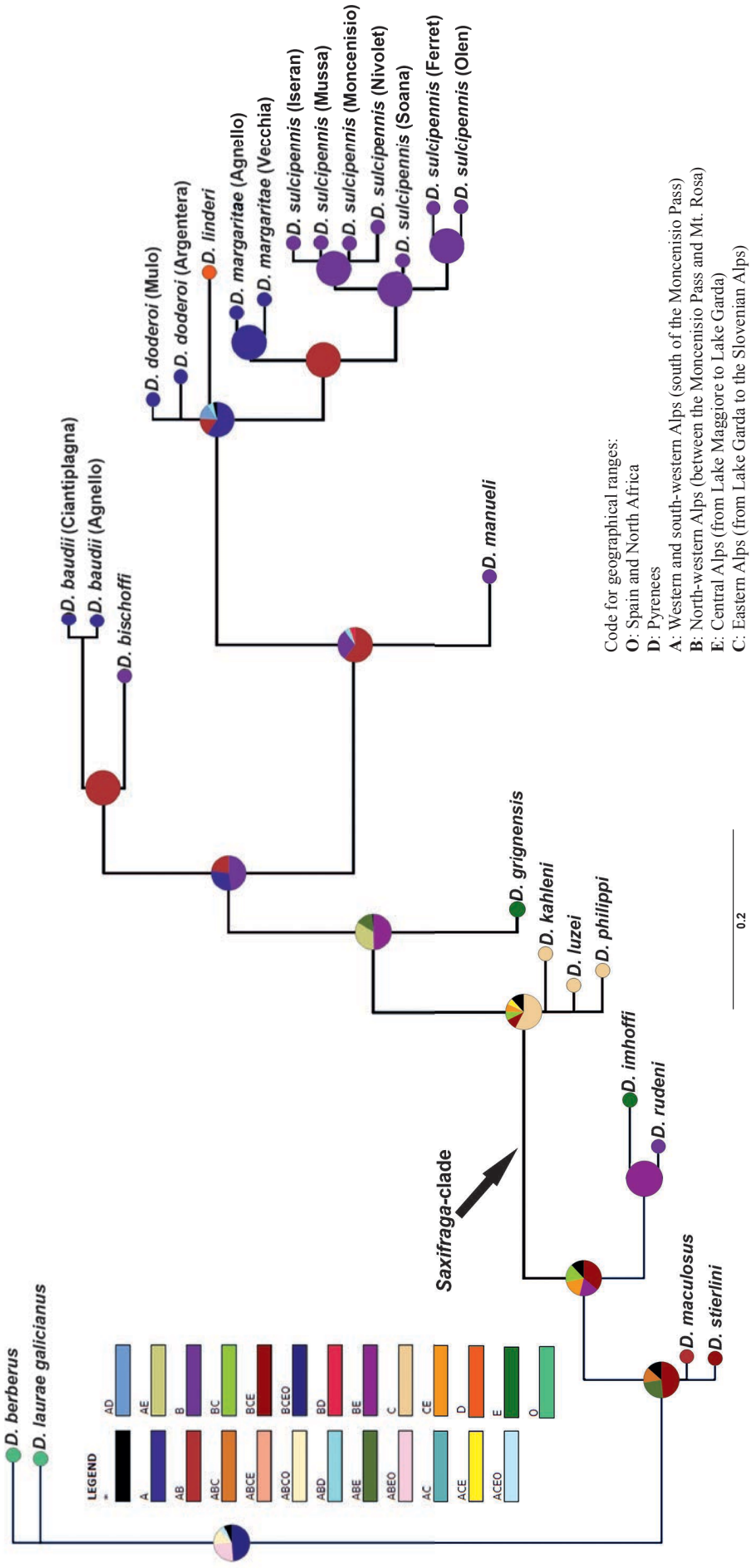


Fig. 57. Geographical range of ancestral species plotted on BI consensus tree from morphological analysis (majority rule extended). The ancestral range as inferred by S-DIVA analysis is reported in the circles.

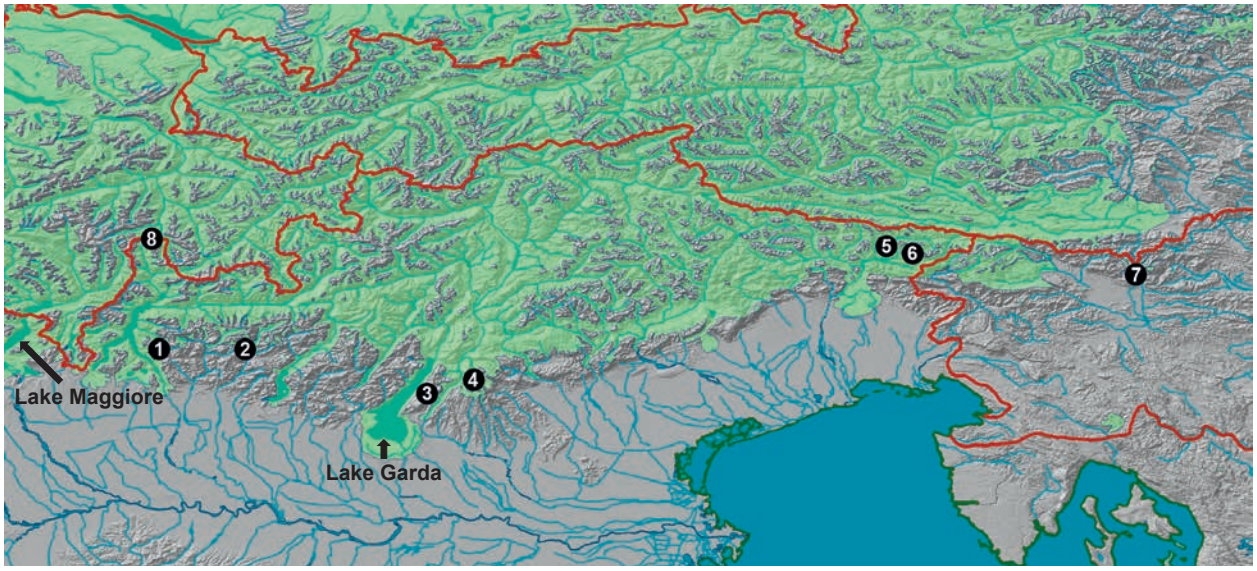


Fig. 58. Limits of the last glacial maximum (green shading) and Nunataks (grey areas surrounded by green shading) in the central and eastern Alps. Collecting localities cited in the text: **1**, Mt. Grigna; **2**, Pizzo Arera; **3**, Mt. Baldo; **4**, Mt. Pasubio; **5**, Mt. Sernio; **6**, Mt. Plauris; **7**, Mt. Grintovec; **8**, Mt. Spluga. Map taken from EHLERS et al. (2011), modified by H. Kühtreiber (Tiroler Landesmuseum Ferdinandeum, Innsbruck, Austria) and further modified. Final image processed using Photoshop CS3 (Adobe Systems Inc.).

4.2. Alpine vs. Iberian and North-African small-sized species

The small-sized, moss-associated species from the alpine region that we have examined clustered as a paraphyletic group around the base of a general alpine lineage also including the large-sized *Saxifraga*-associated lineage, being farther away from the Spanish and North-African species. The present study was not designed to reconstruct phylogeny of the whole genus, the small-sized species were used as outgroup taxa in the morphological analysis, and indeed we have selected only a few of the several characters that present different states among them, namely, those associated with the genital sclerite, and a few other peculiar morphological traits. The small-sized alpine species have in particular a broad and short rostrum and a thicker vestiture of scales and setae on dorsum of rostrum compared to the Iberian and North-African ones (characters 3–5, Figs. 13, 14). The *Saxifraga*-associated species present the same same states of these two characters as the small-sized alpine taxa (Figs. 15, 16).

4.3. The species-group *D. rudeni* – *D. imhoffi*

Our morphological analysis suggests, with moderate support, that the small-sized *D. rudeni* and the large-sized *D. imhoffi* (both feeding on moss) form a clade, derived from among the small-sized taxa and sister to the large-sized *Saxifraga*-associated species. These two species were never considered to be phylogenetically related, despite a general resemblance, and *D. imhoffi* was included in a species-group on its own (OSELLA 1968). The shape of the aedeagus – albeit quite variable in length in *D. imhoffi* (GERMANN 2010) – is indeed very different, as is body size, but *D. rudeni* and *D. imhoffi* have a virtually identical shape of the male genital sclerite (Figs. 29–31), of the pronotum, and of the very broad lobes of the third tarsomere (Fig. 19) (characters 7–13, 22). The lobes are slightly broader than in *D. stierlini*, although we did not score them differently in the matrix; had we done so, the support to their branch would probably have been greater. *Dichotrachelus rudeni* and *D. imhoffi* have also a series of densely placed, erect ovate setae on the suture at the declivity (Fig. 25; character 15), a feature not present in any other *Dichotrachelus*. Most likely, the large size of *D. imhoffi* is homoplastic with respect to the other large-sized species. Both species have larval stages developing on mosses (*D. imhoffi*: GERMANN 2010; *D. rudeni*: MEREGALLI 1980). It appears justified to refer *D. imhoffi* to the *D. rudeni* species-group, rather than maintaining it in an isolated position.

4.4. *Saxifraga*-associated species of *Dichotrachelus*

The *Saxifraga*-associated species, which are all limited to high altitudes, form an almost fully supported clade. The shift to *Saxifraga* may have occurred in the late Miocene, as indicated by the data on the molecular clock (Fig. 56). This time scale is congruent with the late Miocene main uplift of the Alps (FRISH et al. 1998; see also MORENO et al. 2008 for indirect evidence of the palaeoaltitude of the

Eastern Alps based on a palinologic study), and supports the hypothesis that the shift from moss to *Saxifraga* allowed exploitation of the new ecological niche that became available with the uplift of the lands inhabited by the weevils. According to the phylogenetic inference and the S-DIVA, this ecological shift had probably occurred in an area corresponding to the central or the eastern Alps (result for polytomy at base of *Saxifraga*-clade in Fig. 57).

4.5. *Saxifraga*-associated species of the eastern Alps

The *Saxifraga*-associated species present three main species-groups, which differ in their distribution and morphology, and, in part, host-plants. One of these includes three taxa native to the Alps east of Lake Garda: *D. luzei*, *D. kahleni* and *D. philippi*. Meregalli (1992) noticed that the genital sclerite of these species shows a morphology intermediate between the small-sized and the large-sized species, i.e. it appears as overall plesiomorphic for the *Saxifraga*-associated lineage. Their reciprocal relationships could not be ascertained with sufficient support and were not univocal among BI, MP and ML. They were placed in an unresolved polytomy in BI (majority rule 50%), with all other *Saxifraga*-associated species together forming a fourth lineage. However, in BI (majority rule extended) the three species originated from dichotomies in the following sequence: *D. kahleni*, *D. luzei* and *D. philippi*, this last sister to the strongly supported clade (88% pp) with the remaining *Saxifraga*-associated species. So it could be advocated to combine these three species in one "species-group" representing an evolutionary "grade" – as a preliminary solution justified by their overall resemblance, until their reciprocal relationships are fully understood.

Dichotrachelus luzei, *D. kahleni* and *D. philippi* all have a relict distribution, each species being known from one or a few populations restricted to some of the glacial refugia on calcareous bedrocks at the southern margin of the last glacial maximum, or to mountains corresponding to former nunataks by side of, or surrounded by, deeply glaciated valleys, as delimited by Schönwetter et al. (2005) and Ehlers et al. (2011). *Dichotrachelus luzei* is probably endemic to Mt. Grintovec in north-western Slovenia (Fig. 58: 7); *D. kahleni* is also known from a single mountain, Mt. Sernio in the Carnic Alps, though with a closely related form – yet morphologically slightly differentiated (called *D.* cf. *kahleni* herein) – found on the nearby Mt. Plaris in the Julian Alps (Fig. 58: 5, 6); and *D. philippi* was described from Mt. Pasubio and is also known from the nearby Mt. Baldo (Fig. 58: 3, 4). No species of this group were recorded from calcareous mountain ridges between the known localities – but the difficulty in finding these weevils and incomplete field research should be taken into account, so more populations or (sub)species may be present.

The available biological data indicate *Saxifraga caesia* L. as the host plant for *D. kahleni* (Meregalli & Osella 2007) and *D. luzei* (Meregalli, personal observation), *S. paniculata* Mill. for *D.* cf. *kahleni* on Mt. Plaris (Meregalli & Osella 2007) and *S. tombeanensis* Engl. for *D. philippi* on Mt. Baldo (Kahlen, personal observation). All these plants are chasmophytes colonizing calcareous rocky slopes of high altitude (García-González 2008), and the exclusive association of these highly stenoecious *Dichotrachelus* with these saxifrages is the reason for their isolation in the highest parts of the mountains, and the consequent impossibility for them to expand their range.

There is no evidence of Quaternary climate fluctuations having influenced speciation within this species-group, which is an older event. However, these fluctuations may have reduced the ranges of the species and have isolated the single populations. Yet, a glacial or post-glacial speciation of the now isolated populations is occurring, as indicated by the presence of the moderately differentiated and reciprocally isolated forms of *D. kahleni* and *D. philippi* previously cited (the "cf." in Table 1).

By contrast, some of the small-sized moss-associated species distributed in the central to/or eastern Alps, such as *D. vulpinus* Gredler, 1857, have a much wider distribution that also reaches mountains in the northern and north-eastern Alps, and the same is true for several other small-sized species, such as *D. rudeni* and *D. maculosus* in the western Alps. This is due to the widespread availability of *Grimmia* and other mosses that can be used as food-plants, present in variable habitat conditions and with a much greater altitudinal range, so that no geographic barriers existed after glaciation to prevent these species from expanding their range, starting from refuge massifs and nunataks. *Dichotrachelus* of these species were even found on mosses growing in a shaded position on man-made structures, such as house walls or bricks on the sides of paths (Meregalli, personal observation).

4.6. *Dichotrachelus grignensis*, *D. baudii* and *D. bischoffi*

The radiation of the *Saxifraga*-associated species proceeded towards the central and western Alps. The only species known from the central Alps, between Lake Maggiore and Lake Garda, is *D. grignensis*. It is known from the Grigna and, with a relatively distinct form of not yet evaluated taxonomic status, from the Pizzo Arera (Fig. 58: 1, 2), both calcareous massifs. *Saxifraga caesia* is the host plant of *D. grignensis* on Pizzo Arera (Kahlen, personal observation). This species together with *D. baudii* and *D. bischoffi* forms another evolutionary grade, mainly characterized by a very similar morphology of the genital sclerite of the aedeagus (Figs. 36–44). In contrast to the eastern species, however, we obtained morphology-based

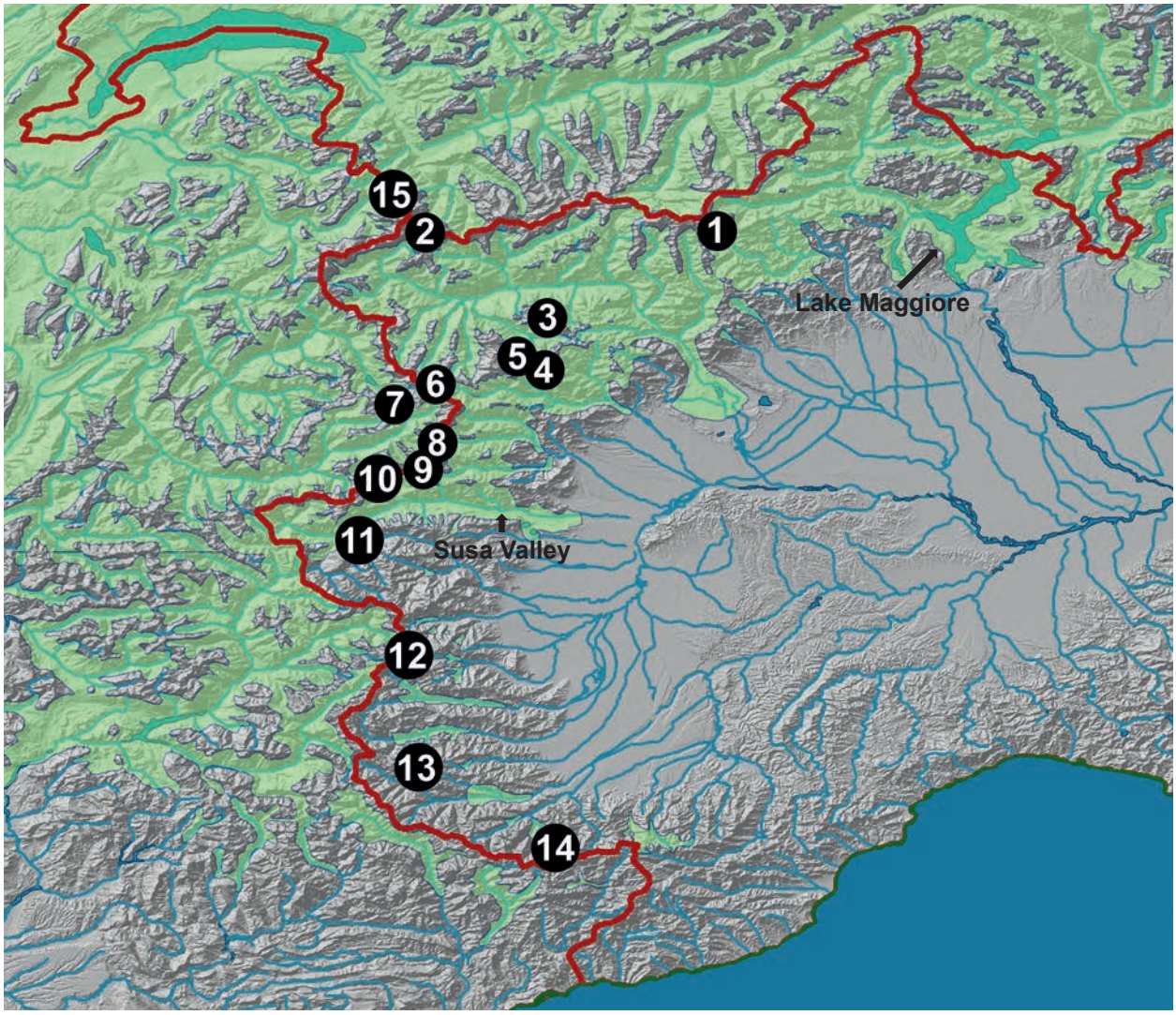


Fig. 59. Limits of the last glacial maximum in the western Alps and Nunataks (shading as in Fig. 58). Collecting localities cited in the text: **1**, Col d'Olen; **2**, Col Ferret; **3**, Val Clavalité; **4**, Val Soana; **5**, Colle Bardoney; **6**, Col Nivolet; **7**, Col Iseran; **8**, Pian della Mussa; **9**, Malciaussia and Mt. Rocciamelone; **10**, Moncenisio; **11**, Colle della Vecchia and Cima Ciantiplagna; **12**, Colle dell'Agnello and Mt. Maniglia; **13**, Col del Mulo; **14**, Lago Brocan and Colle Fremamorta (Mt. Argentera range); **15**, Col de Balme. Map taken from EHLERS et al. (2011), modified by H. Kühtreiber (Tiroler Landesmuseum Ferdinandeum, Innsbruck, Austria) and further modified. Final image processed using Photoshop CS3 (Adobe Systems Inc.).

phylogenetic resolution for this grade, though with only moderate support (Fig. 54): *D. grignensis* + ((*D. baudii* + *D. bischoffi*) + remaining western species). The molecular-based analysis, however, is inconsistent with this, especially with regard to the placement of *D. baudii*.

For the clade comprising the species of the central and western Alps the S-DIVA method did not yield any information on the range of the last common ancestor of this clade, which could be located in any of the western-to-central sectors of the Alps (areas A, B and/or D) that we have defined, though clearly under the inclusion of the central Alps (Fig. 57). The ancestors of both the western clade, after the branching of *D. grignensis*, and of the *D. baudii* + *D. bischoffi* clade were ascribed to the western Alps (areas A and/or B) with 100% likelihood.

Dichotrachelus baudii in the molecular analysis occupied a completely different position, branching off independently from among the small-sized species (Figs. 54 vs. 55). The morphological study of *D. baudii* proved to be quite complex, since this species is characterized by a series of autapomorphies that set it apart from all the other *Dichotrachelus*. In particular, scales and elliptical setae are transformed into hair-like setae, and sculpture is much shallower. Due to its peculiar appearance, it was originally described in a different genus, *Trachelomorphus* Seidlitz, 1875, which was even assigned to a different subfamily (Leptopiinae, now Entiminae Alophini). However, the shape of the genital sclerite is nearly identical between *D. baudii* and *D. bischoffi*, and also details of the sculpture of the integument (below the scales), such as shape and density of the punctures on pronotum, are nearly identical among *D. baudii* and *D. bischoffi* (and *D. manueli*). Hence, in this case we consider the topology derived from the morphological analysis to represent more accurately

the affinities among the species, also taking into account the very different statistical support in the molecular analysis between BI and ML for the origin of *D. baudii* from a further basal dichotomy (respectively, 98% pp vs. 54% bs for the sister taxon of *D. baudii*). After its differentiation, *D. baudii* underwent a notable anagenetic evolution, with acquisition of several autapomorphic traits that separate it from all the other *Dichotrachelus*; only the shape of the genitalia and some non-genital characters reveal its relationships on a morphological basis (see also Meregalli 1992). A long anagenetic evolution is suggestive of an antique separation, and this is independently indicated by palaeogeological information.

D. grignensis represents a geographical, morphological and ecological "intermediate" between the eastern taxa and *D. baudii* & *D. bischoffi*, and it seems likely that the common ancestors of *D. grignensis* and the western alpine *Dichotrachelus* inhabited calcareous massifs in the central Alps. An association with *Saxifraga caesia* and related species and consequent adaptation to limestone outcrops, as also found in the eastern *D. luzei*, *D. kahleni* and *D. philippi*, thus seems to be the "plesiomorphic" condition for the entire *Saxifraga*-associated clade. The last common ancestor of the western alpine clade seems to have undergone an ecological key innovation that promoted the evolution and radiation of the western alpine large-sized species: it apparently was the first *Dichotrachelus* taxon to have become associated with a broader range of *Saxifraga* species, such as *S. oppositifolia*, a widespread perennial herb adapted to several different habitats and soil types, both calcareous and non-calcareous, and *S. bryoides*, a south-European high altitude herb typical of nival screes on usually siliceous substrate (Webb & Gornall 1989). Consequently, they have gained the capability of also colonizing non-calcareous habitats.

Dichotrachelus baudii is known from the Orsiera massif and some localities at the head of Valle Maira (Colle dell'Agnello, Colle Maniglia; Fig. 59: 11, 12). It has always been found on *S. oppositifolia* (Colle della Vecchia and Cima Ciantiplagna: Meregalli & Menardo, personal observation and Meregalli 1992; Colle Maniglia: Menardo, personal observation). *Dichotrachelus bischoffi* is restricted to the Gran Paradiso massif (Fig. 59: 3, 5), where it is relatively frequent above 2500 m altitude and has been found on *Saxifraga bryoides* (Colle Bardoney; Menardo, personal observation) and *S. oppositifolia* (Colle dell'Arietta; Osella 1968, as "*D. manueli*"). The reciprocal distribution of the two species is perfectly congruent with two of the main geological crystalline units of the Western Alps, respectively the mainly non-calcareous Dora-Maira Unit and the Gran Paradiso Unit (Malusà et al. 2005: fig. 1). Together with their relict status, this indicates a speciation by vicariance, very probably determined by the Miocene uplift of the two geological units (Vernon et al. 2008). The 95% confidence interval of the molecular clock is too broad to allow precise remarks, but the mean divergence age for ancestors of *D. baudii* and *D. bischoffi*, about 7 My, is congruent with this scenario.

4.7. *Dichotrachelus manueli*

The remaining species, located in the western Alps except for the Pyrenean *D. linderi*, form an almost fully supported clade in all analyses. According to the S-DIVA reconstruction, the range of the ancestral species was more probably located in the (north-)western Alps, rather than further south (Fig. 57).

Dichotrachelus manueli is a relict species, strictly endemic to Mt. Rocciamelone, a mainly non-calcareous massif, and its nearest northern surroundings (Fig. 59: 9). It is sister to the strongly supported clade that includes *D. sulcipennis*, *D. margaritae*, *D. doderoi* and *D. linderi* (see following sections). Morphologically *D. manueli* is partly intermediate between the previous grade and the monophyletic, more recently differentiated clade around *D. sulcipennis*; in particular, the sclerite of the endophallus in *D. manueli* has a general structure similar to that of the *D. grignensis* – *D. baudii* – *D. bischoffi* grade, but the lateral arms are distinctly longer and more slender, strongly downwards curved – an autapomorphy. However, the setae on the underside are long, dense, and prominent forwards, as in the *D. sulcipennis* group (Figs. 45–53). It feeds on *Saxifraga oppositifolia* (Mt. Rocciamelone, Meregalli & Menardo, personal observation) and is usually found at altitudes higher than 2600 m, even reaching 3300 m; this species probably differentiated in the Rocciamelone region, and remained isolated in the higher parts of this sector of the Alps during glacial periods; its ecological requirements prevented expanding its range to habitats of lower altitude in the mountains north of the Mt. Rocciamelone.

4.8. The *Dichotrachelus sulcipennis* group

Dichotrachelus sulcipennis, *D. margaritae*, *D. doderoi* and *D. linderi* form a strongly supported monophyletic group and share the synapomorphy of a very peculiar shape of the sclerite of the endophallus, unique throughout the genus, with shortened tectum, very narrow, slender arms and median horn, and lateral valves short, not surpassing the proximal 2/3 of the structure (Figs. 48–53). They feed on *Saxifraga oppositifolia* (all species; Meregalli, personal observation), *S. bryoides* (*D. doderoi* at Colle di Fremamorta; Menardo, personal observation) and probably also *S. paniculata* (Kahlen, personal observation for *D. doderoi*). All these *Dichotrachelus* species are known from several different populations, and some have a relatively wide distribution, particularly *D. sulcipennis*. This may have been favoured by the more continuous presence of conspicuous stands of these saxifrages, in particular *S. oppositifolia*, on various lithological substrates, usually metamorphic or crystalline, occasionally calcareous, from 2000 to over 3500 m in altitude, compared with the more or less completely

isolated calcareous outcrops of the central and eastern Alps where *S. caesia* thrives.

Within this group, there is a moderately supported *D. margaritae* + *D. sulcipennis* clade, while otherwise relationships have remained ambiguous (basal polytomy; Fig. 54); individual species are well delimited, but the two included populations of *D. doderoi* (currently referred to distinct subspecies) are not obtained as a phylogenetic unit. A relationship between *D. linderi* and *D. doderoi* was not supported, and in the majority rule extended we did not obtain a concordant topology among BI, ML and MP. We expect clearer results especially on *D. linderi* and *D. doderoi* as soon as gene sequences from the respective populations become available. We note that following a "traditional" morphological approach, a separation of the two *D. doderoi* subspecies (both native to the Maritime Alps) into separate species would not appear justified, whereas there is no doubt that the Pyrenean *D. linderi* is specifically differentiated from *D. doderoi*: *D. linderi* is morphologically differentiated from the two subspecies of *D. doderoi* by 6 and 7 character states, respectively, among those considered here, 5 of these being autapomorphies of *D. linderi*.

This *Dichotrachelus* clade has a more or less continuous distribution all along the western and north-western Alps, from Mt. Rosa to the Maritime Alps, and also occurs in the eastern Pyrenees. This distribution contrasts with one of the most common models of distribution of the south-western alpine – Pyrenean species, which proposes the Susa valley (Fig. 59) to represent the northern-most, insurmountable limit of distribution. The latter type of distribution is typical of taxa differentiated in the Provence-Corsica landmass, often during the early Miocene, and associated with low-altitude forests. Many of these taxa are endogean, such as, among the weevils, the *Raymondionymus* of the *R. fossor* group (MEREGALLI et al. 2006), or some Carabidae (CASALE & VIGNA-TAGLIANTI 1992; VIGNA-TAGLIANTI 1999) and Cholevidae (GIACHINO & VAILATI 1993). In the case of the *Dichotrachelus*, S-DIVA fully supported the last common ancestor of the species of this clade ranging in the north-western and western Alps (Fig. 57), so the Susa valley simply promoted vicariance between *D. margaritae* south of the valley (Fig. 59: 11, 12) and *D. sulcipennis* north of it (Fig. 59: 1, 2, 4, 6, 7, 8, 10). Nowadays the southern-most population of *D. sulcipennis* reaches, but does not go beyond, the Moncenisio area (Fig. 59: 10) whereas the northern-most population of *D. margaritae* lives in the Orsiera ridge (Fig. 59: 11). This vicariance, according to the molecular clock, dates between the late Miocene and the early Pliocene, thus preceding the Quaternary glaciations, and was determined by isolation on high altitude ridges on the sides of the Susa valley during their uplift (see PERRONE et al. 2013 for a survey on the uplift of the Cottian Alps). Absence from localities in between, at the head of the Susa valley, along the main axis of the alpine chain, such as the Monginevro pass, may be due to glaciations, but it should be noticed that there is always a gap where no populations of this *Dichotrachelus* group are known between the limits of distribution of two neighbouring species.

The south-western Alps are inhabited by *D. doderoi*, with two quite differentiated forms (Fig. 59: 13, 14); there is no apparent geographic barrier to separate *D. margaritae* from *D. doderoi vignai*, yet again no populations have been detected between the southern-most known population of *D. margaritae* (Colle dell'Agnello, Fig. 59: 12) and the northern-most population of *D. doderoi vignai* (Colle del Mulo, Fig. 59: 13), despite the presence of the required *Saxifraga* species. In this case, *D. margaritae* is endemic to the Dora-Maira Unit, a mainly crystalline massif, whereas *D. doderoi vignai* is present south of this unit. These species survived the glaciations in refugia located along the eastern and southern sides of the main alpine chain (see SCHÖNSWETTER et al. 2005: fig. 1), or in nunataks surrounded by glaciers, and had a local post-glacial re-colonization, similar to what was demonstrated with a species of *Saxifraga*, *S. florulenta* Moretti, endemic to the Maritime Alps (SZÖVÉNY et al. 2009).

The localities where *Dichotrachelus baudii* was found span across the same range as known for the distribution of *D. margaritae* (it expands slightly further south; Fig. 59: 11, 12 for both). However, as far as known, they are only sympatric at the limits of their range, the Orsiera range at north and the Colle dell'Agnello at south. In intermediate localities (Mt. Albergian) only *D. margaritae* was observed (Meregalli, personal observation). If this were confirmed by further field research, it would mean that the very few known populations of *D. baudii* are old relicts that remained isolated at the extremes of their originally continuous range after the Pliocene and Pleistocene climatic events, and that since then some constraints have prevented *D. baudii* from expanding its range to re-colonize the whole Dora-Maira Unit.

Dichotrachelus margaritae, as well as *D. sulcipennis* and *D. doderoi*, can occasionally be found also at lower altitude, so they have a slightly higher dispersion capability following the host-plant, resulting in a more continuous presence in the Dora-Maira Unit for the former, and in a relatively wider distribution for *D. sulcipennis* and *D. doderoi*.

4.9. *Dichotrachelus sulcipennis*: infraspecific taxa

Dichotrachelus sulcipennis is currently considered to be composed of three different subspecies: *D. sulcipennis sulcipennis* from the Mt. Rosa and surrounding ridges (Fig. 59: 1), *D. sulcipennis bernhardinus* native to the north-western part of the Alps (north-western Piedmont and Aosta Valley; Fig. 59: 2, 4), and finally the southern-most subspecies, *D. sulcipennis pedemontanus* from the Cottian Alps (Fig. 59: 6, 7, 8, 10; OSELLA 1968). The morphology of populations from each of these subspecies was investigated herein. The results allow prelimi-

nary remarks, but many more specimens and populations should be analyzed to achieve sounder results. The distribution of *D. sulcipennis* corresponds well to last-glacial ice-free nunataks (Fig. 59), so it seems that the Quaternary glaciations caused its subspecific fragmentation and that a partial re-colonization occurred in the current post-glacial.

Dichotrachelus s. sulcipennis, here represented by the population from Col d'Olen, and *D. s. bernhardinus* from Col Ferret were very closely related and clustered independently from the other populations examined, hence the taxonomic validity of *D. s. bernhardinus* is in doubt. The population from the Gran Paradiso (Val Soana), originally named *D. tenuirostris* Stierlin, 1878 is presently referred to *D. s. bernhardinus* (cf. Osella 1968), but it clustered in a relatively isolated position, and in ML it was more closely associated with the various populations of *D. s. pedemontanus* rather than with *D. s. bernhardinus*, with a 61% bootstrap support. Hence, from our study it seems that it should be more properly referred to *D. s. pedemontanus*, or kept as a further valid subspecies. The greater affinity of the population from the Soana Valley with those from the Cottian Alps belonging to *D. s. pedemontanus*, and not with those from the mountains north of the Dora Baltea valley, which hosted a deep glacier, is congruent with the relatively continuous glacial refugia in nunataks protected from ice in the western Alps (Fig. 59: 4 and 6–10). *Dichotrachelus s. pedemontanus* has a more or less continuous range between Mt. Bianco and the Susa valley, but it is probably fragmented in several metapopulations, rather than being diffused with a single panmictic population. Minor morphological differences exist between specimens from the extremes of the range, an expected consequence of the inverse ratio between distance and gene flow in these poorly vagile weevils. An evaluation of the infraspecific taxonomy of *D. sulcipennis* was not within the scope of the present paper, thus at this stage no nomenclatorial changes are proposed, pending the examination of several more populations.

4.10. *Dichotrachelus doderoi* and *D. linderi*

For these taxa we only have morphological data. Differentiation between the populations referred to *D. doderoi* present on the mountains of the Maira valley and those from the Maritime Alps was confirmed by our analysis. In the matrix there is a single feature distinguishing them, namely the distinctness of the lobes of the 3rd tarsomere (character 22). Close relationships of *D. linderi* with *D. doderoi*, suggested, but not statistically supported, by some analyses, reflect one of the most frequent biogeographical models of distribution, the presence of links between the south-western Alps and the eastern Pyrenees. These are often considered to have a recent, post-glacial origin (Schmitt 2009). The presence of *D. linderi* in high altitude habitats of the eastern Pyrenees might thus be explained as a colonization following the post-glacial re-colonization of the Pyrenees by the host-plant, *Saxifraga oppositifolia* (Abbot & Comes 2003). Considering the many morphological differences between *D. linderi* and *D. doderoi*, however, the divergence between the two species probably does not date from such very recent times. It might be more convincingly speculated that the last common ancestor to the two taxa could cross southern France and reach the eastern Pyrenees through ice-free but cold areas, with peri-nival vegetation, during one of the glacial periods, not necessarily the last one. If this were the case, *D. linderi* may have differentiated from *D. doderoi* during warmer inter-glacial periods in the early Pleistocene by vicariance in different parts of the range of the ancestor.

4.11. A biogeographical scenario

Fig. 60

Our results on the phylogeny of the alpine species of *Dichotrachelus* and inferences on their biogeographical history indicate the following scenario: the genus, whose nearest relationships are not known, adapted to association with mosses and then started to radiate in the early Miocene, or late Oligocene, in forest habitats, as discussed by Meregalli (1987). The shift to *Saxifraga* occurred in the central or eastern alpine region, probably in the first part of the late Miocene, in concomitance with the final uplift of the chain that resulted in the development of the alpine coenoses, above the timber line. Following this shift, a second radiation occurred that gave rise to several lineages, now restricted to high altitude habitats in the Alps. The initial use in this lineage of the chasmophyte *Saxifraga caesia* resulted in the strict association of these weevils with the calcareous massifs of the central and eastern Alps. Subsequently, radiation of the *Saxifraga*-associated *Dichotrachelus* proceeded towards the western Alps, where the most recent dichotomies within the genus occurred. This radiation was combined with dispersal favoured by adaptation to *Saxifraga* species not restricted to limestone. The late Miocene orogeny caused vicariance among the taxa earlier derived, such as *D. baudii*, *D. bischoffi* and *D. manueli*, now present as relict species endemic to a single geologic unit, whereas the most recently derived taxa of the *D. sulcipennis* group often have a wider distribution. The Quaternary glaciations had less dramatic effects in delimiting the present-day areal in the western Alps than in the central and eastern Alps; the climatic fluctuations do not seem to have had a direct effect on speciation, which had previously occurred, excepting possibly for the vicariance between *D. linderi* and *D. doderoi*, and for the infraspecific differentiation of *D. sulcipennis*. The glaciations however contributed to adaptation to the highest altitude habitats, which remained free from ice, determined a contraction of the range of each species, and led to the reciprocal isolation of the populations of

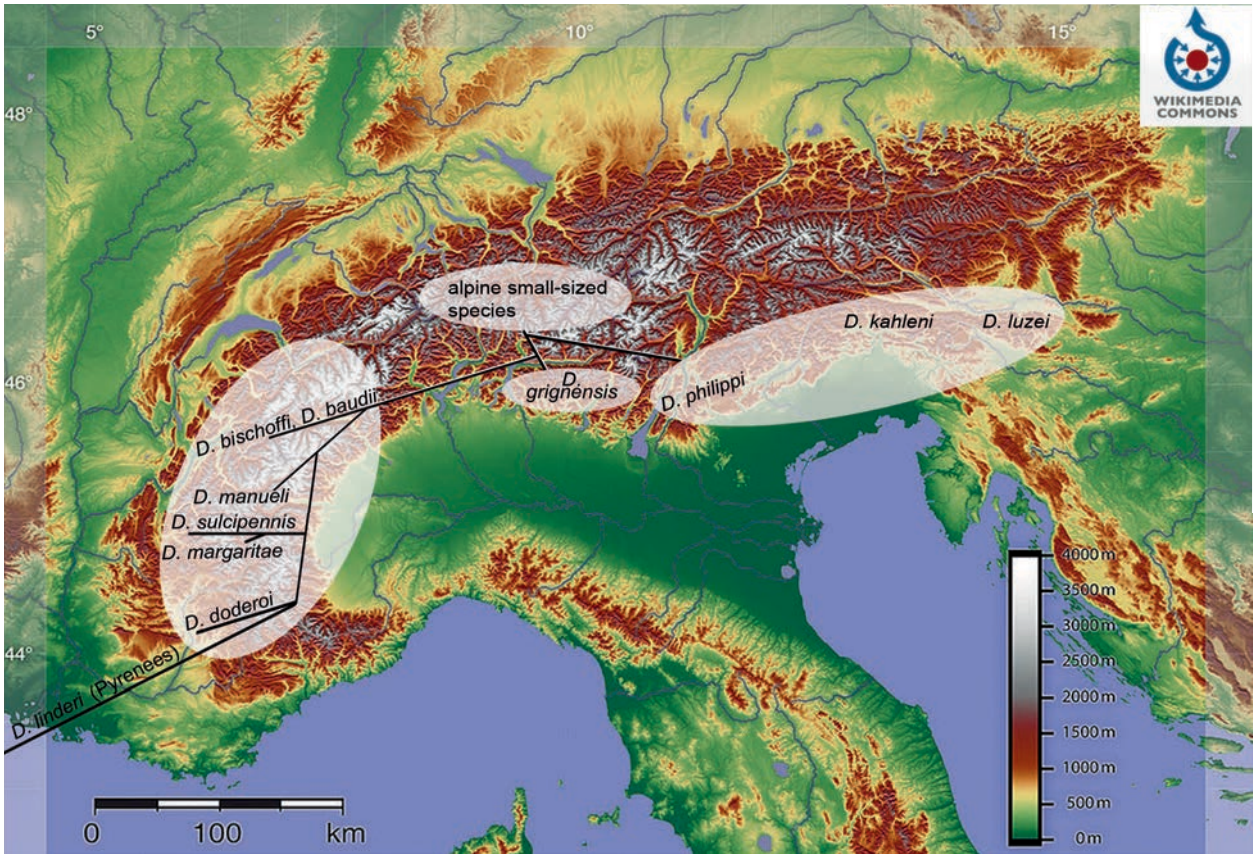


Fig. 60. Phylogeography of the *Saxifraga*-associated species of *Dichotrachelus* plotted on a map of Alps. The tree of the morphology-based phylogenetic inference was simplified for graphic clarity. Map of the Alps taken from Wikimedia Commons (http://commons.wikimedia.org/wiki/File:Alpenrelief_01.jpg, accessed March 25, 2013). Final image processed using Photoshop CS3 (Adobe Systems Inc.).

some species, leading to potential future further speciation. Due to the relatively higher local dispersion capability of the species of the *D. sulcipennis* group, a partial post-glacial re-colonization following the host-plants could occur, leading to the actual range, composed of several metapopulations, as with *D. sulcipennis*, or with a nearly continuous distribution along the whole range, as is apparently the case for *D. margaritae*.

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File: MeregalliEtAl-CurculionidaeDichotrachelus-ASP2013-1.fas: Alignment block of the mtCOI sequences used in the phylogenetic analyses.