

# GENETIC DIFFERENTIATION AND SHELL MORPHOLOGY OF *TROCHULUS OREINOS* (WAGNER, 1915) AND *T. HISPIDUS* (LINNAEUS, 1758) (PULMONATA: HYGROMIIDAE) IN THE NORTHEASTERN ALPS

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## ABSTRACT

*Trochulus oreinos oreinos* and *T. oreinos scheerpeltzi* are two land snail taxa endemic to the Northeastern Austrian Alps, which have been regarded as subspecies of the highly variable, widespread land snail *T. hispidus*. We analysed these three taxa morphologically and genetically to evaluate whether a delimitation between them is possible and, if so, to resolve their phylogenetic relationships. Shell morphological results revealed high similarity between the two *T. oreinos* taxa, and that they are clearly separated from *T. hispidus*. Additionally, the *T. oreinos* subspecies concur with respect to their habitat preferences, as they are both restricted to rocky high alpine areas, whereas the local form of *T. hispidus* is distributed over a wider altitudinal range in moist areas and scrubby perennial herb vegetation near water bodies. While the morphological and ecological results allow clear differentiation between *T. hispidus* and *T. oreinos* only, analyses of the mitochondrial cytochrome *c* oxidase subunit I and 16S rRNA genes revealed high sequence divergences between all three taxa, which indicates that they represent old lineages. The two *T. oreinos* taxa appear as distantly related sister groups, well separated from *T. hispidus*. Whether *T. o. oreinos* and *T. o. scheerpeltzi* should be considered as species cannot be decided at the current state of knowledge.

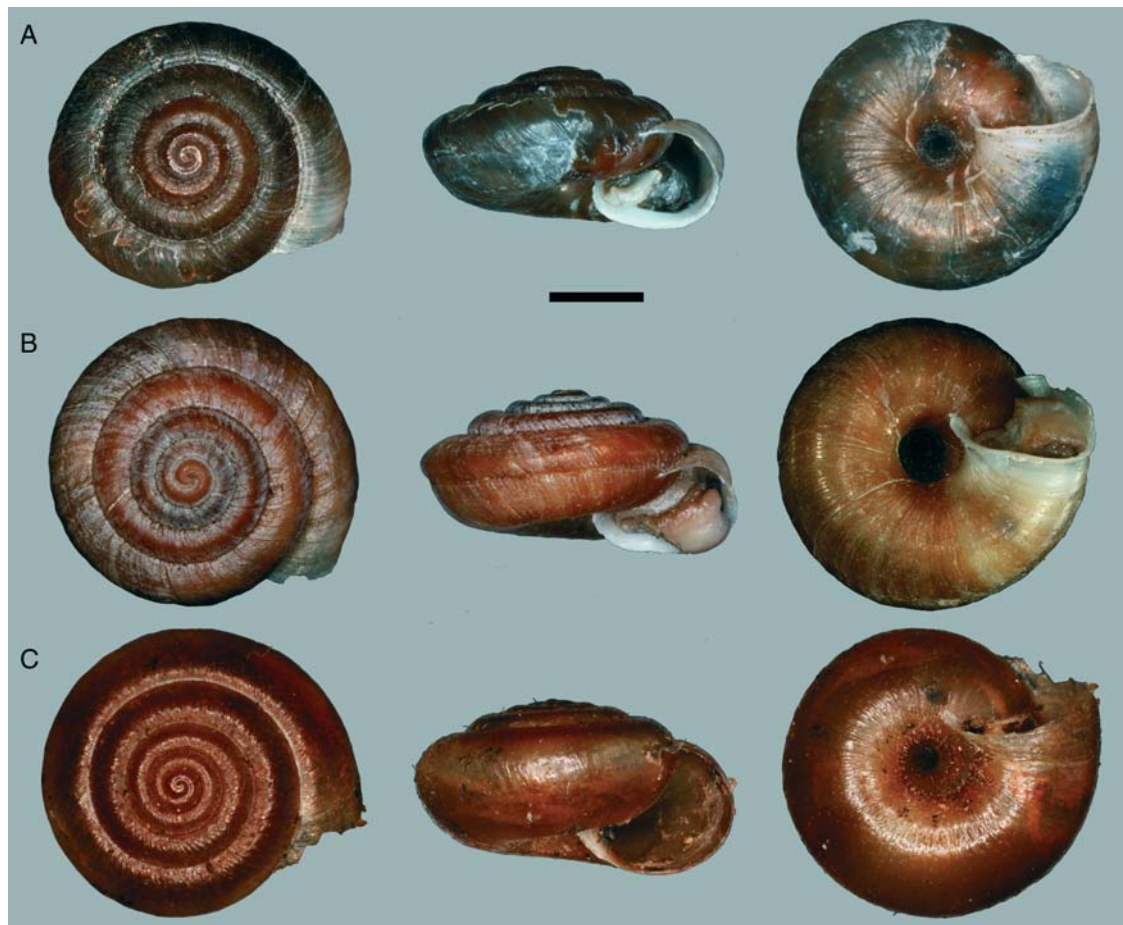
## INTRODUCTION

A large number of land snail species have been described from the Alpine region, including several endemics. Many species have been divided into different infra- or subspecific entities (races, forms etc.), mainly by minor shell morphological features. This differentiation has often been explained by glacial/postglacial events of isolation, displacement, dispersal and (re-)colonization (Adensamer, 1937; Klemm, 1974; Gittenberger, 1991). Moreover, the environment may have triggered special genetic adaptations and/or changes caused by phenotypic plasticity, e.g. smaller shells at higher elevations. Often, subspecific entities have been described from defined altitudinal zones or within the boundaries of particular mountain massifs. Different interpretations and opinions concerning the origin of described morphological variants often resulted in controversial taxonomic conclusions and systematic assignments.

The genus *Trochulus* Chemnitz, 1786 is one such example. The genus comprises small, pulmonate land snails with flattened or globular, particularly hairy shells, mainly distributed in Central and Western Europe. The CLECOM list (Falkner, Bank & von Proschwitz, 2000) gives up to 18 different species of *Trochulus s. s.* for central and northern Europe. Two species – *Trochulus waldemari* (Wagner, 1915) and *T. suberectus* (Clessin, 1878) – were not included by Falkner *et al.* (2000), because their distribution ranges are not covered by the CLECOM list.

However, the number of recognized species varies among different authors. For example, one of the listed taxa, *T. alpicola* (Eder, 1921), has not been regarded as an independent species by some authors. Additionally, some authors (e.g. Davis, 2004; Cameron *et al.*, 2006) have expressed objections to some taxonomic decisions in the CLECOM list. Subsequently, Proćków (2009) enumerated 22 species in *Trochulus* by lumping *T. plebeius* and *T. hispidus* at species level and the (sub)genera *Petasina* and *Plicuteria* at generic level, based on morphological data.

This study is focused on the poorly known northeastern Alpine endemic *T. oreinos* (Wagner, 1915), which was originally regarded as regional subspecies of *T. hispidus* (Linnaeus, 1758), but was later considered as a separate species not closely related to *T. hispidus* (Falkner, 1982, 1995). According to the current taxonomic view, *T. oreinos* comprises two subspecies, *T. oreinos oreinos* (= *Fruticicola hispida oreinos*) and *T. oreinos scheerpeltzi* (Mikula, 1957) (= *T. hispidus scheerpeltzi*), which are both endemic to the northeastern Alps in Austria (Fig. 1A, B). Falkner (1982, 1995) based his decision to treat *T. o. scheerpeltzi* as a subspecies of *T. oreinos* on ‘differing hair morphology’ of the two *T. oreinos* taxa. However, he did not provide any details. This is remarkable because the original description of Wagner (1915) characterized *T. o. oreinos* as hairless, and Mikula (1957) did not mention any hairs in *T. o. scheerpeltzi*. Because no clear descriptions and pictures are available, knowledge of *T. o. oreinos* and *T. o. scheerpeltzi* has been



**Figure 1.** **A.** *Trochulus o. oreinos*, specimen from sample site 34. **B.** *Trochulus o. scheerpeltzi*, specimen from sample site 45. **C.** *Trochulus hispidus*, specimen from sample site 2. Scale bar = 2.0 mm.

restricted to a few specialists who have inspected specimens collected by the describers and deposited in scientific collections.

*Trochulus o. oreinos* (Fig. 1A) is found in Lower Austria and Styria at high altitudes (1,600–2,280 m). Its distribution extends from Schneeberg mountain to Totes Gebirge (Klemm, 1974). Reischütz & Reischütz (2009) mentioned rocky grass biotopes and duff as habitats. In the original description Wagner (1915) characterized it by a shiny, finely granular, hairless shell with coarse, irregular ridges. The shell was described as smaller than that of *T. hispidus*, but ‘more stable’. As additional traits Wagner mentioned a strong lip inside the aperture, visible as a yellow structure from outside, with a tooth-like structure at the basal margin. The type locality is at Hochschwab mountain in Styria at elevations above 2,000 m. So far, no picture of *T. o. oreinos* has been published.

*Trochulus o. scheerpeltzi* (Fig. 1B) is found in the mountain ranges of Hölleengebirge to Totes Gebirge and in parts of Haller Mauern (Klemm, 1974). Like *T. o. oreinos* this subspecies is found at high altitudes (1,600–2,300 m) and in a similar habitat – rocky grass biotopes and crevices with duff (Reischütz & Reischütz, 2009). In the original description Mikula (1957) mentioned a groove beneath a clearly visible keel as a trait distinguishing *T. o. scheerpeltzi* from *T. o. oreinos* and *T. hispidus*. The type locality of *T. o. scheerpeltzi* is ‘Hauptkar’ at the Hohe Nock Mountain in Upper Austria at elevations of 1,600–1,800 m. The only published pictures are those in the original description (Mikula, 1957).

*Trochulus hispidus* (Fig. 1C) has a wide distribution in Europe, occurring over a broad range of altitudes (up to 2,300 m) and habitats. The distribution covers large parts of Europe from Ireland and France to Kazan and St Petersburg in European Russia. In the north it reaches the Arctic Circle. It does not occur in southernmost parts of Europe (Ložek, 1956). According to Giusti & Manganelli (1987) records from Sardinia are very likely due to confusion with *Ichnusotricha berninii*. As *T. hispidus* is a polymorphic species, its systematics have long been the focus of controversy. Forcart (1965) suggested a division of *Trichia hispida* (nowadays *Trochulus hispidus*) into two distinct species, *Trichia hispida* and *Trichia concinna*. Subsequently, he assigned the two subspecies *oreinos* and *scheerpeltzi* to *T. concinna*. However, Gittenberger, Backhuys & Ripken (1970), followed by various authors including Klemm (1974), Falkner (1982) and Naggs (1985), rejected this theory because large clinal transition zones exist between *hispida* and *concinna* and the geographic distribution ranges are not clearly delimited. Shileyko (1978) raised doubts about the justification of the species status of several taxa of the ‘*T. hispida* group’ (including *T. plebeia*, *T. sericea*, *T. septentrionalis* and *T. concinna*). Recent papers that have employed molecular biological methods (Pfenninger *et al.*, 2005; Dépraz, Hausser & Pfenninger, 2009) have provided an even more confusing picture, showing several distinct mitochondrial lineages in *T. hispidus*, and perhaps the occurrence of cryptic species. Proćków (2009) synonymized *T. plebeius* and *T. concinnus* with *T. hispidus* based on an extended morphological analysis.

The questions we wanted to clarify were: (1) Can the three taxa be differentiated morphologically? (2) Are the two *T. oreinos* taxa and the northeastern Alpine form of *T. hispidus* genetically differentiated? (3) Do the morphological and genetic data corroborate the species status of *T. oreinos*? (4) Do the three forms occupy different habitats, elevations and geographic ranges? Using samples covering the entire distribution range of *T. o. oreinos* and *T. o. scheerpeltzi* as well as the local forms of *T. hispidus* we performed morphological and genetic analyses to answer these questions.

The systematics of some species of *Trochulus* are problematic. This is especially true of *T. hispidus* with a number of divergent lineages (Pfenninger *et al.*, 2005; Dépraz *et al.*, 2009), among which there may be some cryptic species. However, since no comprehensive phylogenetic study of *Trochulus* has yet been carried out, we provisionally adopted the classification of the Austrian taxa provided by Reischütz & Reischütz (2007).

## MATERIAL AND METHODS

### Study area and sampling

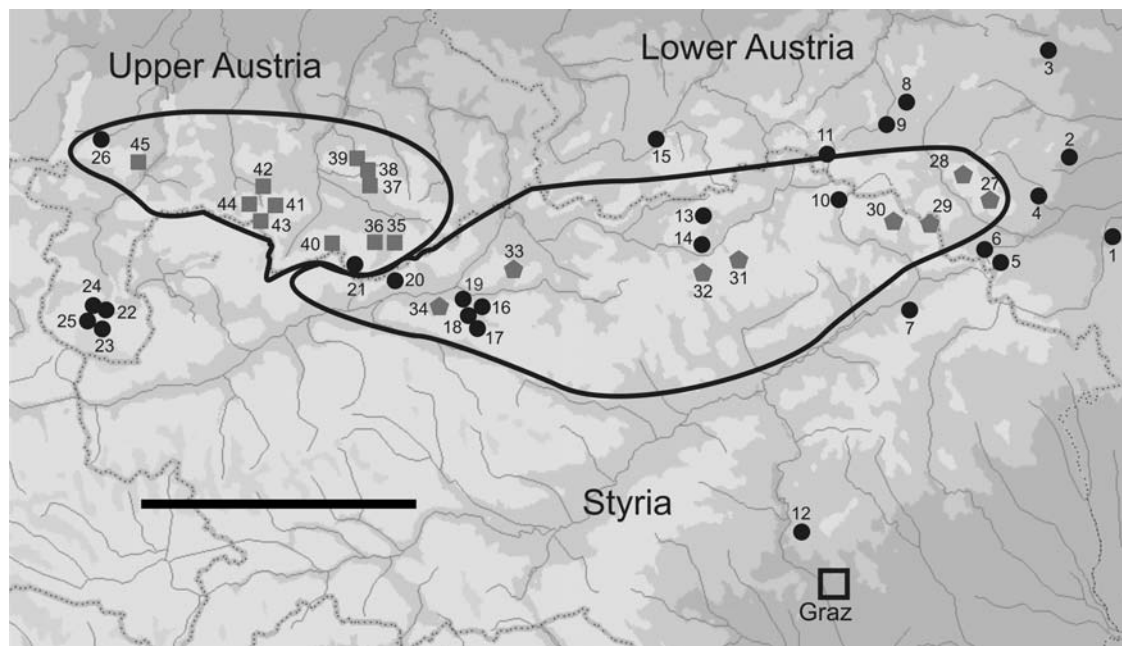
The study area was located in the Northeastern Austrian Alps, including parts of the provinces Upper Austria, Lower Austria and Styria. This covers the distribution ranges of the two *T. oreinos* taxa (according to Klemm, 1974) and adjacent areas where *T. hispidus* occurs (Fig. 2). Most sample sites are situated on the limestone bedrock of the Northern Calcareous Alps, one on Palaeozoic limestone of the Grazer Bergland and four on metamorphic rocks of the Central Alps. Most of the study area is characterized by a cool humid Central European climate with heavy precipitation; only the easternmost and southeastern parts are influenced by the warmer and dryer Illyrian and Pannonian climates (Kilian, Müllner & Starlinger, 1994). Both *T. oreinos* subspecies occur down to an altitude of 1,300–1,450 m, which is also the lower limit of the subalpine zone. Like other marginal Alpine areas, the study area has been the

focus of research projects seeking potential glacial refugia (e.g. Schönswetter *et al.*, 2005), because large parts of it remained ice-free during the last glaciations (Van Husen, 1997).

Sampling and habitat analysis were carried out at 45 sampling sites (Fig. 2, Table 1). Topotypes of *T. o. oreinos* (11 specimens from sample site 32) and *T. o. scheerpeltzi* (11 specimens from sample site 38) were included. Exact positions and elevations of collection sites were determined using GPS. The sampling period extended from May to July in 2007, 2008 and 2009. Adjacent water bodies, vegetation, habitat structure and dominant plant species were recorded. *Trochulus hispidus* was identified by morphological traits described in the literature (e.g. Ložek, 1956; Gittenberger *et al.*, 1970; Kerney, Cameron & Jungbluth, 1983). *Trochulus o. oreinos* and *T. o. scheerpeltzi* were identified using the original descriptions (Wagner, 1915; Mikula, 1957) as well as by comparison with reference specimens (paratypes and syntypes) in the collections of the Natural History Museum, Vienna (NHMW). In total, 327 specimens (225 living animals and 102 empty shells) of the three *Trochulus* taxa were included (Table 1).

### Shell morphology

Four shell traits were measured in intact adult specimens (shell diameter, umbilicus diameter, shell height and height of last whorl) with a graduated eyepiece in a stereomicroscope. The definition of adulthood from shell apertural traits was problematic, as presumably adult specimens of *T. hispidus* often lack an outer lip (Geyer, 1915; Frömming, 1954; Cameron, 1982). Therefore, individuals were defined as ‘adult’ when their shells had a minimum diameter of 5.4 mm, as this was the size of the smallest individual seen with a fully developed lip. This might appear arbitrary, because the standard literature and some collections contain only ‘typical’ specimens with outer lip. However this analysis was intended to include all naturally occurring variants and therefore we had to define a size limit. Altogether 304 specimens (complete specimens and empty shells) from all 45 sample sites were measured and standard



**Figure 2.** Sample localities: *Trochulus o. oreinos*, grey pentagons; *T. o. scheerpeltzi*, grey squares; *T. hispidus*, black circles. Numbers correspond to localities in Table 1. Distribution ranges of *T. o. oreinos* and *T. o. scheerpeltzi* (according to Klemm, 1974) are delineated with black lines. The scale bar represents 50 km.

DIFFERENTIATION OF *TROCHULUS OREINOS* AND *T. HISPIDUS*

**Table 1.** Sampling localities of *Trochulus* taxa.

Species	Number of locality	Name of locality	E	T	L	M	S	J	G	A
<i>T. hispidus</i>	1	Pittental-Schlattenbach	397	3	3	2	0	1	3	2
	2	Würflach-Johannesbachklamm	445	5	3	4	2	1	3	2
	3	Berndorf-Grabenweg	412	6	6	6	0	0	3	3
	4	Sierningtal-Stixenstein	470	9	4	9	5	0	3	3
	5	Semmering-Maria Schutz	871	3	3	2	0	1	3	2
	6	Breitenstein-Adlitzgraben	650	3	2	3	1	0	2	2
	7	Fischbacher Alpen-Hauereck	1,187	2	2	1	0	1	2	1
	8	Halbachtal-Rosbachklamm	649	5	5	5	0	0	3	3
	9	Tiefental-Ochbauer	739	10	10	10	0	0	3	3
	10	Frein-Freinbach	869	10	10	10	0	0	3	3
	11	Göller-Gscheid	914	10	10	10	0	0	3	3
	12	Grazer Bergland-Semriach	503	11	10	11	1	0	10	10
	13	Dürradmer-Kräuterin	1,100	3	3	3	0	0	3	3
	14	Salzatal-Weichselboden	660	3	3	2	0	1	3	3
	15	Dürrenstein-Lechnergraben	604	3	3	2	0	1	3	2
	16	Johnsbachtal-Wasserfallmauer	978	5	3	5	2	0	3	3
	17	Johnsbachtal-Köblwirt	868	3	3	3	0	0	3	3
	18	Johnsbachtal-Kneippstation	865	3	3	2	0	1	3	2
	19	Johnsbachtal-Langriesmündung	652	3	3	2	0	1	3	2
	20	Großer Phyrgas-Arlingsattel	1,425	10	10	10	0	0	2	3
	21	Warscheneck-Wurzeralmbahn	810	1	1	1	0	0	1	1
	22	Hallstatt-Salzberg	942	3	3	3	0	0	3	3
	23	Hallstatt-Klausalm	796	4	3	3	1	1	3	2
	24	Hallstatt-Sportplatz	524	3	3	2	0	1	3	2
	25	Hallstatt-Waldbachstrub	806	4	4	4	0	0	4	4
	26	Hochlecken-Taferlklaue	778	1	1	1	0	0	1	1
<i>T. o. oreinos</i>	27	Schneeberg-Waxriegel	1,873	41	3	41	38	0	3	3
	28	Schneeberg-Fadenwände	1,562	7	2	6	5	1	2	1
	29	Rax-Bismarksteig	1,787	36	6	31	30	5	6	1
	30	Schneealpe-Schauerkogel	1,664	11	9	11	2	0	3	3
	31	Hochschwab-Severinkogel	2,010	1*	1	0	0	0	1	0
	32	Hochschwab-Schiestlhaus	2,179	11	11	10	0	1	3	2
	33	Tamischbachturm	1,940	10	1	10	9	0	3	3
	34	Admonter Kalbling	2,026	9	6	9	3	0	6	6
<i>T. o. scheerpeltzi</i>	35	Großer Phyrgas-Haller Mauern	1,900	11	11	10	0	1	3	2
	36	Großer Phyrgas-Westgrat	2,000	3*	3	2	0	0	3	3
	37	Hohe Nock-Feichtausee	1,399	10	10	10	0	0	2	2
	38	Hohe Nock-Hauptkar	1,704	11	11	11	0	0	3	3
	39	Hohe Nock-Haltersitz	1,583	10	10	10	0	0	3	2
	40	Warscheneck-Toter Mann	2,028	2	1	2	1	0	1	1
	41	Großer Priel-Welser Hütte	1,747	9	9	8	0	1	3	2
	42	Großer Priel-Hinterer Ackergraben	1,564	2	2	2	0	0	2	2
	43	Großer Priel-Schlund	2,284	2	2	2	0	0	2	2
	44	Großer Priel-Fleischbanksattel	2,157	12	12	10	0	2	3	1
	45	Höllengebirge-Bledigupf	1,677	3	1	3	2	0	1	1
			327	225	304	102	21	132	111	

Abbreviations: E, elevation (metres above sea level); T, total number of investigated specimens; L, total number of living specimens (adult and juvenile); M, total number of morphologically investigated specimens (adult; living and empty shells); S, total number of adult empty shells; J, total number of living juvenile specimens; G, total number of genetically investigated specimens (selected living adult and all juvenile specimens); A, total number of specimens investigated genetically and morphologically. \*One adult living specimen of each of these samples was broken.

variables (mean, variance and standard deviation) were calculated. Hairs (which are present also in juveniles) were inspected in all 327 individuals. Another two adult specimens could be investigated only genetically, because their shells were broken. A total of 111 specimens were investigated using all methods (Table 1). The measurement error is too often neglected in measurements of small (<10 mm) globular shells. The main source of error is the lack of precise measurement

points on the shell. Furthermore, the definition of the main axes is not very precise and the projection of the shell in two dimensions is problematic. Measurement error was determined by repeated measurements (10 times) of shell diameter, umbilicus diameter and shell height in 140 empty shells of *Trochulus* and examination of the distribution of residuals (total 4,200).

Four additional shell traits were recorded as presence/absence, because they were mentioned as typical for

*T. o. oreinos* in the original description (Wagner, 1915). Three of these were apertural traits: basal tooth, internal rib and paler area around the aperture (Fig. 3). The occurrence of coarse, irregular ridges was also recorded as presence/absence. Ridges were classified as 'coarse' if broad ridges (>0.5 mm) were immediately followed by smaller ones (Fig. 3). In *T. o. scheerpeltzi* a groove beneath the keel was described by Mikula (1957) and was recorded by us in three categories: well developed, partly developed and absent. If the groove was clearly visible with  $\times 16$  magnification and covered at least 50% of the circumference, it was characterized as well developed. If it covered less than this and was only weakly visible, it was characterized as partly developed. Measurements of shells were log-transformed. These data together with scores obtained with a correspondence analysis of presence/absence data were used in a canonical discriminant analysis (Tabachnick & Fidell, 1996). Morphometric analyses were performed with programs written by one of us (H.W.). Since we were interested in identifying group differences and the variables responsible for these differences, we used discriminant analysis rather than PCA or ordination techniques that deal with overall variation, which might be dominated by variables that do not contribute to, or even mask, the variation among groups.

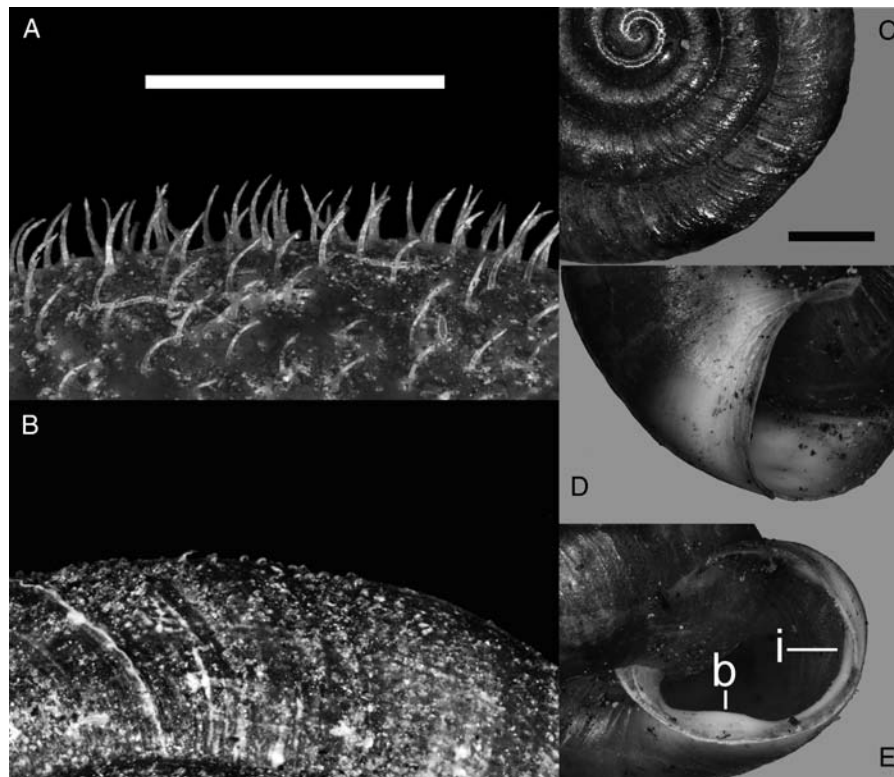
To quantify hair length and structure, digital microscopic images were taken of five hairs of 15 specimens (five of each form). Hair lengths were measured by using TPSdig Version 2.14 (Rohlf, 2001). To proof the repeatability of measurements, all hairs were measured twice.

As measuring of hairs takes a lot of time and the different hair morphologies can be easily recognized (Fig. 3), the values from all specimens were assigned to three categories: long hairs (>0.2 mm), short hairs (<0.1 mm) and no hairs.

#### Genetic analysis

From 132 specimens (adults and juveniles) a partial region of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was sequenced. In addition, from representatives of each clade a partial region of the mitochondrial 16S rRNA (16S) gene was sequenced (altogether 38). As outgroup taxa *Monacha cantiana* and *Plicateria lubomirskii* (one specimen each) were analysed for both fragments.

A piece of foot tissue was extracted with QIAgen Blood and Tissue Kit. Primers were based on those used by Gittenberger, Piel & Groenenberg (2004) for COI and by Pfenninger, Posada & Magnin, (2003) for 16S. Primers were optimized on the basis of alignments of published sequences of several snail species. Primer sequences for COI: COI<sub>folmer</sub> fwd 5'-GGTCAACAATCATAAAGATATTGG-3' (LCO1490 modified from Folmer *et al.*, 1994) and COI<sub>schneckrev</sub> 5'-TATACTTCTGGATGACCAAAAATCA-3' (H2198-Alb modified from Gittenberger *et al.*, 2004). Primer sequences for 16S: 16S<sub>fwd</sub> 5'-CGCAGTACTCTGACTGTGC-3' (Pfenninger *et al.*, 2003) and 16S<sub>sch\_rev</sub> 5'-CG CCGGTCTGAACTCAGATC-3' (16S<sub>rev</sub> modified from Pfenninger *et al.*, 2003). Resulting fragment sizes were 705 bp (COI) and about 395 bp (16S), respectively. PCR was performed on a Master gradient thermocycler (Eppendorf) in 50  $\mu$ l with 1 U *Taq* DNA polymerase (Roche), 1  $\mu$ M of each primer and 0.2 mM of each dNTP (Boehringer Mannheim). Each PCR comprised 35 reaction cycles with the following annealing temperatures: 50°C (COI) and 55°C (16S). Control reactions for both DNA extractions and PCR amplifications were carried out. PCR products were purified using the QIAquick PCR Purification kit (QIAGEN) and analysed with direct sequencing (both directions). Sequencing using the amplification primers was performed by AGOWA (Berlin, Germany).



**Figure 3.** Shell traits. **A.** Hairs of *Trochulus hispidus* (specimen from sample site 17). **B.** Hairs of *T. o. scheerpeltzi* (specimen from sample site 37). **C–E.** *Trochulus o. oreinos* specimen from sample site 29. **C.** Coarse ripples. **D.** Pale area around aperture. **E.** Internal rib (i) and basal tooth (b). Scale bars = 1.0 mm.

For the COI sequences the alignment was straightforward as there were no insertions or deletions. Alignment of 16S sequences was performed with Tcoffee (Notredame, Higgins & Heringa, 2000) and adjusted manually. Neighbour-joining (NJ; Saitou and Nei, 1987) dendrograms were calculated with ClustalX v. 2.0.12 (Larkin *et al.*, 2007) using *p*-distances. Bootstrap analyses were performed with 1,000 replicates. For calculation of models of sequence evolution the sequences were collapsed to haplotypes using Collapse1.2 (Posada, 2004). The resulting dataset was used applying the Akaike information criterion corrected for small sample size (AICc) as implemented in the jModelTest v. 0.1.1. (Posada, 2008); the selected models were HKY + I + G for the COI dataset, GTR + G for the 16S dataset and GTR + I + G for the combined (COI + 16S) dataset. Bayesian analyses (BI) were performed using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001). Runs were started with random trees and performed for 2 million generations each with four Markov chains, and a sampling frequency of every 100th generation. Those trees generated prior to stationarity were discarded as burn-in and were not included in the calculation of the consensus trees.

Numbers of haplotypes and haplotype diversity and average *p*-distances (gaps excluded) were calculated with ARLEQUIN v. 3.11 (Excoffier, Laval & Schneider, 2005). The sequences determined in this study are deposited in GenBank under the accession numbers HQ204370–HQ204503 (COI) and HQ204504–HQ204543 (16S).

## RESULTS

### Shell morphology

*Trochulus hispidus* shows higher variation in all shell measurements than the *T. oreinos* forms, which are at least 0.5 mm smaller (Table 2). Of the four measurements, shell width and umbilicus width differed between the two *T. oreinos* taxa. Although the *T. oreinos* subspecies are smaller and less variable than *T. hispidus*, ranges overlap. Small specimens of *T. hispidus* (e.g. those from sample site 17 at Sierningtal-Stixenstein) overlap the range of *T. oreinos*. Therefore, shell measurements alone are not a suitable discriminating character for the three taxa. Although the systematic measurement error was relatively high (with 1% error probability, from  $\pm 0.14$  to  $\pm 0.18$  mm), it did not compromise these results (see Table 2). Measures showed small differences between *T. o. oreinos* and

*T. o. scheerpeltzi*, but large differences between both taxa and *T. hispidus*.

At first sight *T. hispidus* has remarkably longer hairs than both *T. oreinos* subspecies. Hair length of *T. hispidus* ranges from 0.21 to 0.31 mm (mean 0.27 mm), of *T. o. oreinos* from 0.03 to 0.09 mm (mean 0.06 mm) and of *T. o. scheerpeltzi* from 0.04 to 0.08 mm (mean 0.06 mm). Additionally, hairs of the *T. oreinos* subspecies are often curled or strongly bent, while those of *T. hispidus* are only slightly bent. The problem with this trait is that elder specimens and empty shells often lack hairs. For example, of the 118 specimens investigated in *T. o. oreinos*, 86 were hairless, but of these individuals 78 were empty shells. Among *T. o. scheerpeltzi* individuals 59 out of 70 were hairy, most of them collected alive. Among the 116 specimens of *T. hispidus* 106 showed the characteristic long hairs.

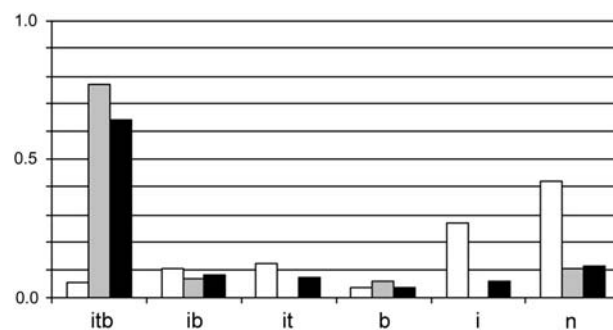
The *T. oreinos* taxa show strong development of the lip and aperture (internal rib, basal tooth, pale around aperture), while in *T. hispidus* these traits occur only occasionally and rarely in combination (Fig. 4). Most specimens of *T. hispidus* show only an internal rib or even none of these traits. Both *T. oreinos* forms consistently show strong irregular ruffles, while only 8 of 116 specimens of *T. hispidus* have this character. The groove beneath the keel proved not to be a constant character of *T. o. scheerpeltzi* as two specimens were found in which this trait was virtually absent. On the other hand, nine specimens of *T. o. oreinos* were found in which a faint groove was present, five of them from site 34 (Tamischbachturm). At this same site three *T. o. oreinos* with a well-developed groove were found.

The occurrence of coarse ruffles could not be included in the discriminant analysis, as it is a constant trait of both *T. oreinos* forms. The variables which most strongly influenced the results of the analysis were the qualitative characters (hair length, groove, internal rib, basal tooth, pale area), especially the apertural traits. The dominance of these factors on the first axis caused a visible 'horseshoe' effect (Fig. 5A). The results show a clear differentiation between *T. o. scheerpeltzi* and *T. hispidus*. *Trochulus o. oreinos* and *T. hispidus* are visibly differentiated but still close. The two *T. oreinos* taxa show an overlap in the discriminant analyses (polygons in Fig. 5A) mainly caused by the occurrence of a groove beneath the keel of some specimens of *T. o. oreinos*. The first axis explains 84% and the second 16% of the total variation. The first axis separates *T. hispidus* from the other populations, and correlates negatively with the scores associated with the first reciprocal ordering component (mainly defined by the groove beneath the keel and hair length), and positively with all linear measurements, indicating a general size difference. *Trochulus o. scheerpeltzi*

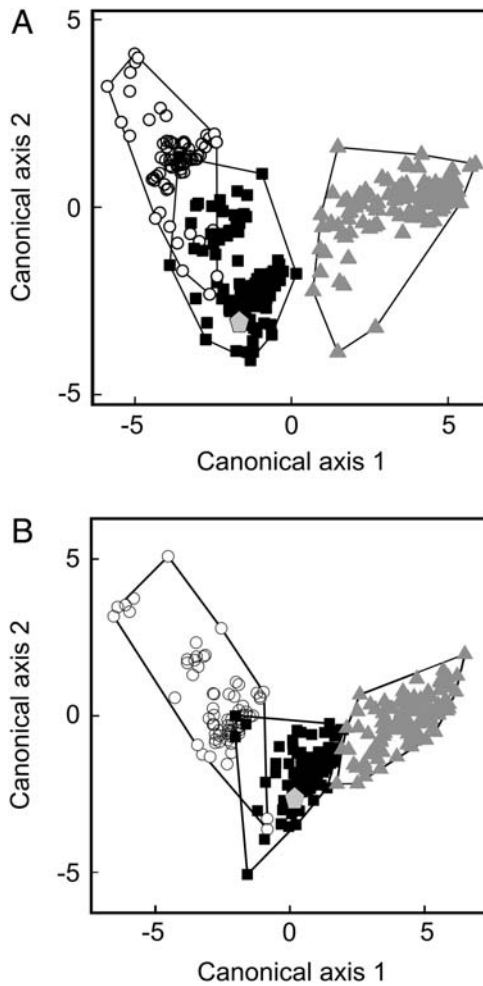
**Table 2.** Summary of shell measurements (mm) of *Trochulus* taxa.

		Range	ME	Mean	SD	SE
<i>T. o. oreinos</i> , 8 sample sites, <i>n</i> = 118	WS	5.4–7.5	0.15	6.37	0.43	0.04
	WU	0.8–1.6	0.14	1.24	0.15	0.01
	SH	2.8–4.2	0.18	3.42	0.28	0.03
	HW	2.0–2.8	0.17	2.40	0.19	0.02
<i>T. o. scheerpeltzi</i> , 11 sample sites, <i>n</i> = 70	WS	5.6–7.5	0.15	6.50	0.38	0.05
	WU	0.9–1.5	0.14	1.16	0.13	0.02
	SH	2.8–4.0	0.18	3.43	0.25	0.03
	HW	2.0–2.9	0.17	2.39	0.18	0.02
<i>T. hispidus</i> , 26 sample sites, <i>n</i> = 116	WS	5.5–8.4	0.15	7.18	0.65	0.06
	WU	1.0–2.3	0.14	1.59	0.22	0.02
	SH	2.9–4.7	0.18	3.79	0.36	0.03
	HW	2.1–3.5	0.17	2.80	0.26	0.02

Abbreviations: WS, width of shell; WU, width of umbilicus; SH, shell height; HW, height of last whorl; ME, measurement error; SD, standard deviation; SE, standard error of the mean.



**Figure 4.** Combination of apertural traits of *Trochulus o. oreinos*, *T. o. scheerpeltzi* and *T. hispidus* in proportion values. White bars, *T. hispidus*; grey bars, *T. o. oreinos*; black bars, *T. o. scheerpeltzi*. itb: internal rib, basal tooth, pale area around aperture; ib: internal rib, pale area around aperture; b: pale area around aperture; i: internal rib; n: none of these traits.



**Figure 5.** Discriminant analysis of *Trochulus o. scheerpeltzi*, *T. o. oreinos* and *T. hispidus*. The outlier was treated separately as we considered it a malformation (see text). **A.** Discriminant analysis including hair traits. Wilks' Lambda =  $4.6328E-02$ ,  $F_{14,590} = 153.7$ ,  $P < 0.00001$ . **B.** Discriminant analysis excluding hair traits. Wilks' Lambda =  $4.2035E-02$ ,  $F_{14,588} = 120.1$ ,  $P < 0.00001$ . Grey triangles, *T. hispidus*; white circles, *T. o. scheerpeltzi*; black squares, *T. o. oreinos*; grey pentagon, *T. hispidus* (outlier).

scores higher on the second axis, which is mainly characterized by a negative correlation with the scores derived from the second axis of the correspondence analysis (mainly defined by strong development of the groove beneath the keel) (Table 3). Hair length could not be analysed in several shells which had obviously lost the hairs (empty shells and some live-collected). However, as this is an essential discriminant trait, we included it in our analysis. An additional discriminant analysis excluding hair length is shown in Figure 5B. It reveals the same groups, although *T. o. oreinos* and *T. hispidus* are less clearly separated from each other.

There is one outlier of *T. hispidus*, a rather small, hairless, empty shell (site 4) which has an internal rib and a pale area around the aperture. This outlier was not included in the computation of the discriminant function, but was subsequently scored to show its position (Fig. 5).

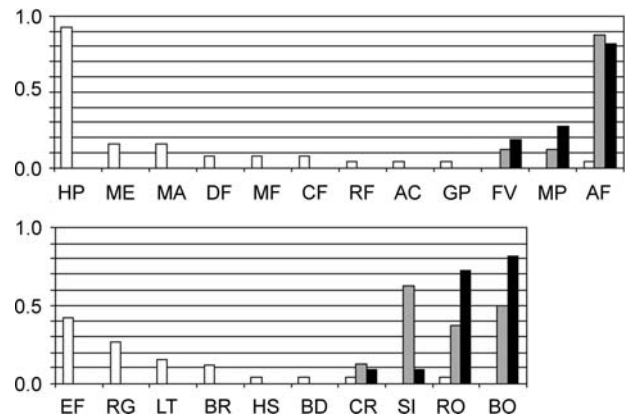
#### Habitat selection and elevation

*Trochulus o. oreinos* was found at elevations from 1,562 to 2,179 m, *T. o. scheerpeltzi* from 1,399 to 2,157 m and *T. hispidus*

**Table 3.** Correspondence analysis of *Trochulus* taxa: linear discriminant coefficients of linear discriminant.

Variable	Linear discriminant coefficients	
	1	2
WS	-0.35081	-0.26637
WU	0.57778	0.11054
SH	0.28020	0.69537
HW	0.47491	0.34116
ROC1	-0.44336	0.05416
ROC2	0.10844	-0.46297
ROC3	-0.17531	0.31563

Abbreviations: WS, width of shell; WU, width of umbilicus; SH, shell height; HW, height of last whorl; ROC1–3, reciprocal order components.

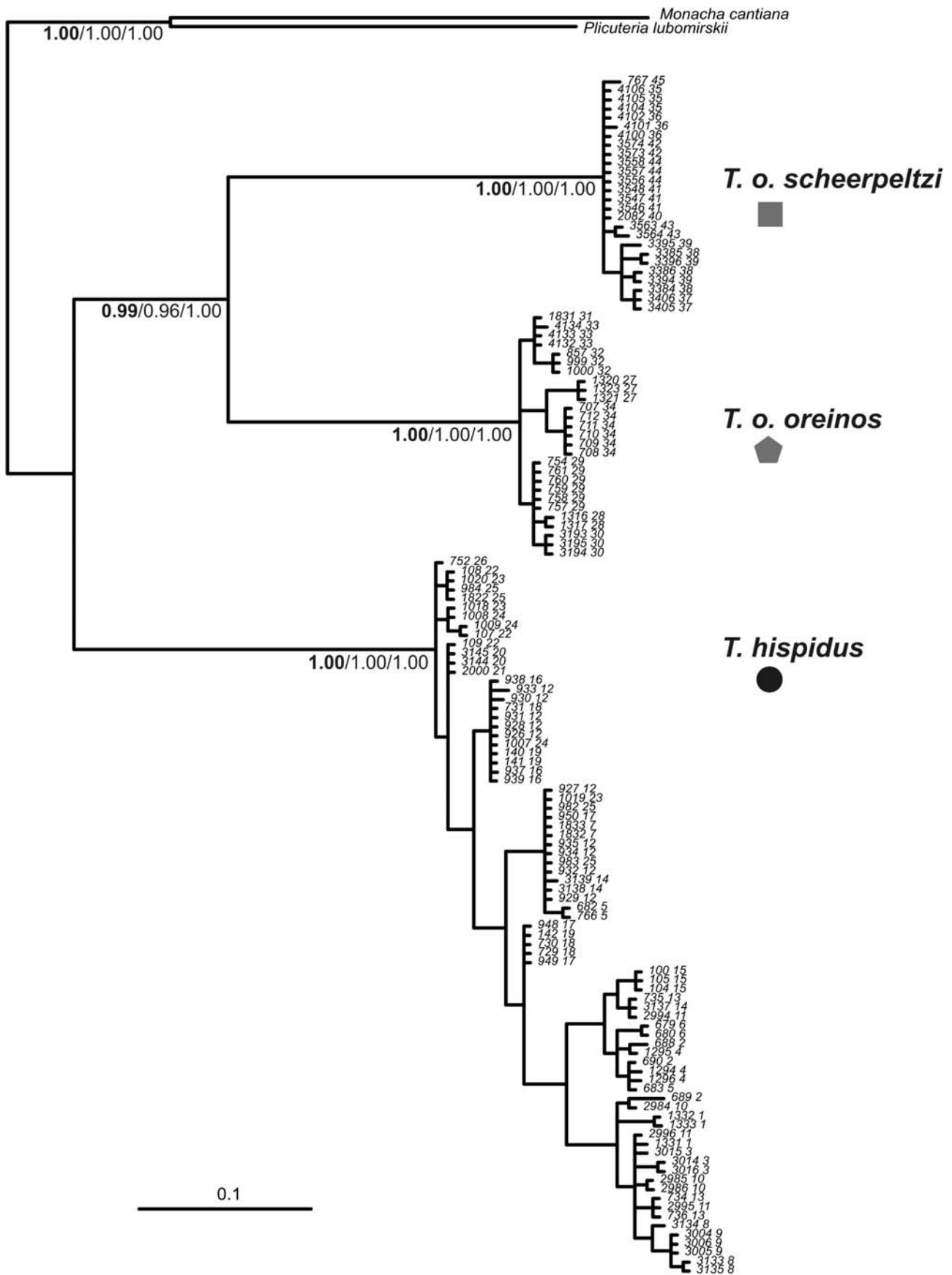


**Figure 6.** Habitat (top) and landscape structure (below) preferences of the three *Trochulus* taxa in proportion values. White bars, *T. hispidus*; grey bars, *T. o. oreinos*; black bars, *T. o. scheerpeltzi*. Abbreviations: HP, high perennial herbs; ME, meadow; MA, marsh; DF, deciduous forest; MF, mixed forest; CF, coniferous forest; RF, riparian forest; AC, alder carr; GP, garden/park; FV, free of vegetation; MP, mountain pine shrubbery; AG, alpine grassland; EF, edge of forest; RG, riverbank grove; LT, loose trees and shrubs; BR, boundary ridge; HS, hedgerows and shrubs; BD, bank/dam; CR, canyon/rock face; SI, single stones; RO, rocks; BO, boulders.

from 397 to 1,425 m (Table 1). Both *T. oreinos* taxa were found to be restricted to rocky habitats, mostly among sparse alpine grass or in vegetation-free areas and mountain pine shrubbery, while *T. hispidus* preferred moist habitats, in particular tall perennial herbs, often near water bodies (Fig. 6). *Trochulus hispidus* inhabits a wider range of vegetation types and landscape structures than both *T. oreinos* forms.

#### Molecular analysis

Among the 132 individuals analysed for COI, 51 different haplotypes were detected. In the 38 individuals from which 16S sequence was obtained, 19 different haplotypes were observed. In the BI tree based on the COI dataset (Fig. 7) three clearly differentiated groups are found: (1) *T. hispidus*, (2) *T. oreinos scheerpeltzi* and (3) *T. oreinos oreinos*. The same topology was obtained from both 16S and combined datasets, and with a different tree-building algorithm (NJ; data not shown). All three taxa are highly supported (maximum posterior probability) in analyses of all three datasets. The two *T. oreinos* taxa are well-supported sister groups in all trees. The genetic distances among the three clades are high. Average  $p$ -distances in



**Figure 7.** Bayesian tree of the COI sequences of *Trochulus* species. Posterior probabilities of the main groups are indicated at the nodes (COI/16S/COI + 16S). Posterior probabilities of the depicted tree (COI) are in bold. Labelling of the individuals include individual number and locality (before the space; as in Table 1). *Monacha cantiana* and *Plicuteria lubomirskii* were used as outgroups.



the COI sequences are 13.3% between the two *T. oreinos* taxa. Between *T. hispidus* and *T. o. oreinos*, and *T. o. scheerpeltzi*, the values are 14.4% and 16.0%, respectively. The average *p*-distance for the 16S sequences is 13.7% between the two *T. oreinos* taxa. Between *T. hispidus* and *T. o. oreinos* and *T. o. scheerpeltzi* the values are 18.0% and 17.4%, respectively. Both *T. oreinos* subspecies have a somewhat lower haplotype diversity (*T. o. scheerpeltzi*: 0.88 and *T. o. oreinos*: 0.70) compared to *T. hispidus* (0.95), but the number of sample sites is different in all three (*T. hispidus* 26, *T. o. oreinos* 8, *T. o. scheerpeltzi*, 11). In *T. oreinos* specimens from one locality had either the same or very similar haplotypes, while in *T. hispidus* quite distinct haplotypes coexist even within one locality (e.g. sample site 25).

## DISCUSSION

The results obtained by the two different approaches (morphological and genetic) agree only in some aspects. The clear differentiation between *Trochulus hispidus* and the two *T. oreinos* taxa is supported by hair and shell morphology, genetic analysis and ecological preferences. In contrast, a clear differentiation between *T. o. oreinos* and *T. o. scheerpeltzi* is only found in the genetic analyses, but not in morphology or ecological preferences.

### *Differentiation between T. hispidus and T. oreinos*

*Trochulus o. oreinos* and *T. o. scheerpeltzi* are very similar to each other shell morphology and ecology, while *T. hispidus* differs from these taxa in both respects and also appears to be more variable. Hair morphology discriminates the *T. oreinos* subspecies from *T. hispidus*, and this character is also useful for juvenile specimens. The basic pattern for hair length and distance between hairs in *Trochulus* is supposed to be a stable trait as it is determined by glands in the mantle (Kaiser, 1966). Other shell morphological traits also show clear differences between *T. oreinos* subspecies and *T. hispidus*. A constant trait of both *T. oreinos* subspecies are the coarse ruffles. Furthermore, only 20% of both *T. oreinos* subspecies, but nearly 70% of *T. hispidus*, did not show fully developed apertural traits. Seasonality cannot explain this observation, as the snails were collected at similar times of the year. A lack of fully developed apertural traits in adult specimens of *T. hispidus* has been reported several times in the literature. Weinland (1883) mentioned that it is difficult to find specimens of *T. hispidus* with a fully developed lip. A similar finding was reported by Geyer (1915), who noticed that in Upper Austrian populations of *T. hispidus* many adult specimens did not show a lip.

Shell measurements alone are not enough for a reliable differentiation. Furthermore, these traits can also be influenced by the environment (Davis, 2004). Our results show that *T. hispidus* is more variable than both *T. oreinos* subspecies. Other authors have also described substantial shell variability in *T. hispidus*, which can result in difficulties in species recognition (Ložek, 1956; Forcart, 1965; Naggs, 1985; Von Proschwitz, 1993; Pročková, 2009). However, in contrast to previous studies, we found no specimens with pronounced globular shells.

With respect to habitat preferences a clear difference was revealed between *T. hispidus* and *T. oreinos*. Both *T. oreinos* subspecies are restricted to rocky areas with alpine vegetation at high elevations, whereas *T. hispidus* extends over a larger range, predominantly in lower areas, preferring moist habitats with a well-developed herb layer, often close to water bodies. The small overlap in the altitudinal distribution of *T. hispidus* and *T. oreinos* subspecies might be due to the geomorphological conditions of the northern Calcareous Alps. Springs and creeks usually discharge at the base of the mountain massifs, while

plateaus and slopes at high elevation remain dry (Lieb, 1991). Where damp habitats extend up to higher elevation, *T. hispidus* can be found above 1,000 m, as at the sampling sites 'Fischbacher Alpen-Hauereck' (1,180 m) and 'Haller Mauern-Arlingsattel' (1,425 m). The wide distribution of *T. hispidus* even in formerly glaciated areas of Austria might be due to the fact that rivers and creeks act as linear corridors along which the snails can easily disperse (actively or passively via rafting). The occurrence of *T. o. scheerpeltzi* at 1,399 m at the site 'Hohe Nock-Feichtausee' is the lowest one ever recorded for this taxon, as the known range is 1,600–2,300 m (Klemm, 1974). Nevertheless, this site fits the habitat preferences of *T. o. scheerpeltzi* because it is an azonal deep habitat of high alpine vegetation resulting from the cool microclimate of a sunless shady slope with northern exposure.

Our genetic data are in accordance with the morphological and ecological differentiation between *T. hispidus* and *T. oreinos*. We cannot conclude that *T. oreinos* and *T. hispidus* are sister species, owing to our limited sampling of other taxa, and because the phylogenetic status and monophyly of *T. hispidus* are unclear (see above). This underlines the need for a complete phylogeny of the genus. The high genetic distances (mean distance for COI 15.2%) (only slightly higher than those between the two *T. oreinos* subspecies: mean distance for COI 13.3%) suggest that the differentiation between the lineages leading to these two species might have taken place before the Pleistocene glaciations. Also, the difference in the morphological variability in *T. oreinos* and *T. hispidus* is reflected in the haplotype variation, *T. hispidus* showing higher haplotype diversity than the other two taxa. The haplotype diversity found within the *T. oreinos* taxa is rather low (0.88 and 0.70). Direct comparison of the haplotype diversity is problematic, since the number of sampled localities is higher in *T. hispidus* than in the two *T. oreinos* taxa, but it is still meaningful since we sampled the whole distribution range of the two *T. oreinos* taxa. Hence, it cannot be expected that the diversity will increase much by including more localities and samples. Only a part of the distribution range was sampled *T. hispidus*, but well-differentiated haplotypes can be found within a single locality. The low haplotype diversity within the two *T. oreinos* taxa is remarkable, since the populations live on isolated mountain peaks. This suggests past extinction and recolonization events, perhaps during glacial periods. Other studies have reported highly divergent lineages within *T. hispidus* (Pfenninger et al., 2005; Dépraz et al., 2009). However, in our analysis all *T. hispidus* from the foothills of the northeastern Austrian Alps belong to a single haplogroup, distantly related to the haplogroups of *T. hispidus* published so far (Pfenninger et al., 2005; Dépraz et al., 2009). Thus, our analysis reveals yet another lineage that morphologically resembles *T. hispidus*.

### *Differentiation between T. o. oreinos and T. o. scheerpeltzi*

While the morphological differentiation between *T. hispidus* and *T. oreinos* is straightforward, the distinction between *T. o. oreinos* and *T. o. scheerpeltzi* is difficult, as there are no apparent differences in hair and shell morphology, size and habitat selection. The only discriminating characters mentioned in the literature are the different geographical ranges and the groove beneath the keel. However, our results indicate that the groove is not a constant trait of *T. o. scheerpeltzi* as some specimens lack this characteristic, whereas specimens with a groove were found at the western sample sites of *T. o. oreinos*. The discriminant analysis (Fig. 5) showed overlap of the two taxa. These findings are in accordance with reports of intermediate forms between the two *T. oreinos* taxa (Mikula, 1957; Falkner, 1970, 1982) and suggest why the two forms have been classified as subspecies. However, from the

molecular results there is no indication of hybridization between *T. o. oreinos* and *T. o. scheerpeltzi*. The distribution of mitochondrial haplotypes is completely in accordance with the distribution ranges of the two taxa; no haplotypes of *T. o. oreinos* were found in the range of *T. o. scheerpeltzi* or vice versa. It could be hypothesized that the groove is just a result of phenotypic plasticity and/or connected with a yet unknown adaptation to climate or habitat conditions. As the distribution ranges of both subspecies belong to the same geological formation, it is unlikely that geological factors could be of importance. In this region there is a transition between two local climatic zones – western vs eastern part of the northern margin of the northeastern Alps (as defined by Kilian *et al.*, 1994). The transition between these two climatic zones covers the eastern part of the distribution area of *T. o. scheerpeltzi* as well as the western part of the *T. o. oreinos* distribution (Fig. 2), i.e. regions where some morphologically atypical individuals (*T. o. oreinos* possessing a groove, *T. o. scheerpeltzi* lacking a groove) were detected. Subtle climate differences might in some way influence the formation of a partially or fully developed groove. Another explanation for the presence of a groove in the specimens from Tamischbachturm is that they are malformations, although it is hard to explain why this should have happened coincidentally to many individuals.

The genetic data present a different picture. *Trochulus o. scheerpeltzi* and *T. o. oreinos* are well separated. The  $p$ -distance values for COI and 16S are surprisingly high. It has been suggested that terrestrial gastropods might have a substantially higher substitution rate in their mtDNA than that reported for other animal groups (Thomaz, Guiller & Clarke, 1996; Hayashi & Chiba, 2000; Davison, 2002; Van Riel *et al.*, 2005). However, this is not a plausible explanation for the surprisingly high distances found between the *Trochulus* taxa, since preliminary analysis of nuclear 5.8S rRNA, ITS2 and 28S rRNA sequences support the hypothesis of an old split (LK, unpublished results). It should be mentioned that many studies report moderate evolutionary rates in molluscs (e.g. Pfenninger & Magnin, 2001; Haase *et al.*, 2003; Ketmaier, Giusti & Caccione, 2006). Hence, our data suggest a long separation of *T. o. scheerpeltzi* and *T. o. oreinos*.

The shell morphological traits and ecological preferences do not provide strong arguments to raise the two subspecies *T. o. oreinos* and *T. o. scheerpeltzi* to species level. They also do not differ in the gross anatomy of the reproductive organs (Mikula, 1957). More detailed anatomical analyses (e.g. cross-sections of penis and vagina) should be performed to search for differences in these traits. Applying the ecological and phylogenetic species concepts, *T. o. oreinos* and *T. o. scheerpeltzi* are not unequivocally separated, as they inhabit the same habitat and no diagnostic morphological traits have been detected so far. The status of *T. oreinos* as an independent species separated from *T. hispidus* (Falkner, 1982) is corroborated by morphological (groove, hairs) and ecological (habitat preferences) arguments. The clear genetic separation corresponding to the geographical distributions did not provide any indication for interbreeding between the two taxa. Nevertheless, in the light of the biological species concept further analyses (e.g. ecophysiological, anatomical, breeding experiments) are needed to test the potential for interbreeding before a final decision can be made about species or subspecies status.

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