Zoologica Scripta

KUNGL. VETENSKAPS-AKADEMIEN
THE ROYAL SWEDISH ACADEMY OF SCIENCES

Paraphyly and budding speciation in the hairy snail (Pulmonata, Hygromiidae)

Luise Kruckenhauser, Michael Duda, Daniela Bartel, Helmut Sattmann, Josef Harl, Sandra Kirchner & Elisabeth Haring

Submitted: 10 April 2013
Accepted: 7 December 2013
doi:10.1111/zsc.12046

Kruckenhauser, L., Duda, M., Bartel, D., Sattmann, H., Harl, J., Kirchner, S. & Haring, E. (2014). Paraphyly and budding speciation in the hairy snail (Pulmonata, Hygromiidae). — *Zoologica Scripta*, *43*, 273–288.

Delimitation of species is often complicated by discordance of morphological and genetic data. This may be caused by the existence of cryptic or polymorphic species. The latter case is particularly true for certain snail species showing an exceptionally high intraspecific genetic diversity. The present investigation deals with the *Trochulus hispidus* complex, which has a complicated taxonomy. Our analyses of the *COI* sequence revealed that individuals showing a *T. hispidus* phenotype are distributed in nine highly differentiated mitochondrial clades (showing p-distances up to 19%). The results of a parallel morphometric investigation did not reveal any differentiation between these clades, although the overall variability is quite high. The phylogenetic analyses based on *12S*, *16S* and *COI* sequences show that the *T. hispidus* complex is paraphyletic with respect to several other morphologically well-defined *Trochulus* species (*T. clandestinus*, *T. villosus*, *T. villosulus* and *T. striolatus*) which form well-supported monophyletic groups. The nc marker sequence (*5.8S–ITS2–28S*) shows only a clear separation of *T. o. oreinos* and *T. o. scheerpeltzi*, and a weakly supported separation of *T. clandestinus*, whereas all other species and the clades of the *T. hispidus* complex appear within one homogeneous group. The paraphyly of the *T. hispidus* complex reflects its complicated history, which was probably driven by geographic isolation in different glacial refugia and budding speciation. At our present state of knowledge, it cannot be excluded that several cryptic species are embedded within the *T. hispidus* complex. However, the lack of morphological differentiation of the *T. hispidus* mitochondrial clades does not provide any hints in this direction. Thus, we currently do not recommend any taxonomic changes. The results of the current investigation exemplify the limitations of barcoding attempts in highly diverse species such as *T. hispidus*.

Corresponding author: *Luise Kruckenhauser, Central Research Laboratories, Natural History Museum Vienna, Burgring 7, 1010 Vienna, Austria. E-mail: Luise.Kruckenhauser@nhm-wien.ac.at*

Luise Kruckenhauser, Natural History Museum Vienna, Central Research Laboratories, Burgring 7, Vienna, 1010, Austria. E-mail: Luise.Kruckenhauser@nhm-wien.ac.at

Michael Duda, 3rd Zoological Department, Natural History Museum Vienna, Burgring 7, 1010, Vienna, Austria. E-mail: Michael.Duda@nhm-wien.ac.at

Daniela Bartel, University of Vienna, Department of Integrative Zoology, Althanstraße 14, 1090, Vienna, Austria. E-mail: Daniela.Bartel@univie.ac.at

Helmut Sattmann, 3rd Zoological Department, Natural History Museum Vienna, Burgring 7, 1010, Vienna, Austria. E-mail: Helmut.Sattmann@nhm-wien.ac.at

Josef Harl, Sandra Kirchner, and Elisabeth Haring, Natural History Museum Vienna, Central Research Laboratories, Burgring 7, Vienna, 1010, Austria and University of Vienna, Department of Integrative Zoology, Althanstraße 14, 1090, Vienna, Austria. E-mails: Josef.Harl@nhm-wien.ac.at, Sandra.Kirchner@nhm-wien.ac.at, Elisabeth.Haring@nhm-wien.ac.at

Introduction

Recognising species as biological entities is, besides the human interest in describing and categorising nature, the basis for many biological investigations. Yet for many species groups, this task is not at all trivial. Cryptic as well as highly polymorphic species may hamper unambiguous species assignment, leaving biologists unsatisfied. This is especially valid for poorly studied species. Molecular genetic approaches brought invaluable progress in understanding the history of populations and hence of speciation. Sometimes, however, the results of molecular genetic analyses reveal inconsistencies between morphological differentiation and phylogenetic relationships based on DNA (mostly mitochondrial) sequences. A case in point is certain snail species exhibiting an exceptionally high intraspecific genetic diversity in mitochondrial (mt) sequences. (Hayashi & Chiba 2000; Haase *et al.* 2003; Van Riel *et al.* 2005; Dillon & Robinson 2008; Davison *et al.* 2009; Scheel & Hausdorf 2012).

The genus *Trochulus* Chemnitz, 1786 represents an example of complicated taxonomy, species differentiation and delimitation. The most common species within this genus, *T. hispidus* (Linnaeus, 1758), is widely distributed in Europe. It occurs over a broad range of altitudes, up to 2300 m above sea level (asl) and habitats. The range covers large parts of Europe from Ireland and France in the west to western Russia (southern Ural) in the east (Ložek 1956; Kerney *et al.* 1983; Sysoev & Schileyko 2009). In the north, it reaches the Arctic Circle, in the south, the northern part of the Iberian Peninsula, the Mediterranean and the Balkan Peninsula. *T. hispidus* is a morphologically polymorphic species and, as a consequence, its systematics has long been a matter of controversy (Forcart 1965; Proćków 2009; Welter-Schultes 2012; Proćków *et al.* 2013), for example, concerning morphologically similar species, as *Trochulus plebeius* (Draparnaud, 1805), *Trochulus sericeus* (Draparnaud, 1801) and *Trochulus coelomphola* (Loccard, 1888) (e.g. Falkner 1982; Proćków 2009).

One in the meanwhile clarified example, *T. oreinos* (Wagner 1915) was originally regarded as a regional subspecies of *T. hispidus*, but was later considered as a separate species (Falkner 1982). In our previous molecular study (Duda *et al.* 2011) on *T. oreinos*, we were able to confirm the latter assumption: The two taxa are morphologically and genetically clearly separated, and the high sequence divergence indicates that they split a long time ago. The genetic investigation, which comprised *T. hispidus* specimens collected in the areas surrounding the distribution range of *T. oreinos*, revealed monophyly for both *T. hispidus* and *T. oreinos* (Duda *et al.* 2011). However, an earlier analysis of *T. hispidus* using samples from Germany, France and Switzerland indicated high genetic variability with various, distinct mitochondrial lineages (Pfenninger *et al.* 2005). This raised the question whether there are additional *T. hispidus* lineages in the Eastern Alps. The taxonomy of *T. hispidus* is further complicated by *Trochulus sericeus*: this species is characterised by a more globular shell and a narrow umbilicus, but turned out to be not clearly differentiated from *T. hispidus* (Pfenninger *et al.* 2005) on the basis of mtDNA sequence data. Therefore, the various lineages and morphotypes have been summarised under the designation *Trochulus hispidus/sericeus* complex (Pfenninger *et al.* 2005). Further investigations of differentiated mtDNA lineages, nuclear (nc) markers and morphology indicated the presence of cryptic species within this complex (Pfenninger *et al.* 2005; Depraz *et al.* 2009). The results of Pfenninger *et al.* (2005) indicate that detailed geographic sampling is crucial for a meaningful interpretation of the phylogeography of this complex. In this context, note that the Austrian *T. hispidus* clade detected in our previous study (Duda *et al.* 2011) was highly differentiated from those described by Pfenninger *et al.* (2005). This finding suggests that the Alpine region might have played an important role for the diversification of the lineages of this complex. This calls for gathering more data from the Eastern Alps and surrounding lowland areas in eastern Austria. These regions are known to have served as glacial refugia for several invertebrates and vascular plants and as a consequence still display high levels of endemism (Tribsch & Schönswetter 2003; Schönswetter *et al.* 2005; Rabitsch *et al.* 2009). In the current study, we performed for the first time an exhaustive analysis of the variability of *T. hispidus* and *T. sericeus* in Austria using mitochondrial and nuclear DNA marker sequences. In addition, we analysed several samples from surrounding countries (Hungary, Italy, Slovenia, Switzerland and southern Germany) as well as from Sweden the type locality of *T. hispidus*, and the Netherlands. We also included several other Central European species of the genus *Trochulus* into the analysis to clarify their phylogenetic relationships and to compare the amount of intraspecific variation. The main objective of the study was to gain more insights into the complicated evolutionary history of *T. hispidus*. By assessing mtDNA variation, we wanted to obtain a clearer picture about the geographic distribution of haplotypes. This should, together with conchological and anatomical analyses, provide a basis to attempt a delineation of *T. hispidus*. Specifically, we addressed the following questions: (i) Are there additional clades with *T. hispidus* or *T. sericeus* phenotype besides those reported in earlier studies (Pfenninger *et al.* 2005; Depraz *et al.* 2009) and are (some of) the latter distributed also in the Alpine region? (ii) What is the

distribution of clades? (iii) Are there regions where clades co-occur? (iv) Is there a pattern in the ncDNA analysis that reflects the results of the mtDNA? and (v) Do the data suggest the presence of cryptic species?

In a parallel approach, the same individuals as in this study were investigated morphologically to determine whether the differentiation found in the mtDNA sequences is accompanied by shell morphological diversification. These data are presented elsewhere (Duda *et al.* revised), but will be discussed in the context of the genetic results.

Material and methods

Study area and sampling

We collected living specimens of the genus *Trochulus* from 126 sampling sites (Fig. 1). Exact positions and elevations of collection sites were determined using GPS. Additional samples from Switzerland, Germany and Sweden were kindly provided by colleagues (Ulrich Schneppat, Ira Richling, Ted von Proschwitz and Zoltan Feher). Snails with a *T. hispidus* and *T. sericeus* phenotype, *Trochulus biconicus* (Eder, 1917), *Trochulus villosus* (Draparnaud, 1805), *Trochulus coelomphala*, *Trochulus clandestinus* (Hartmann, 1821),

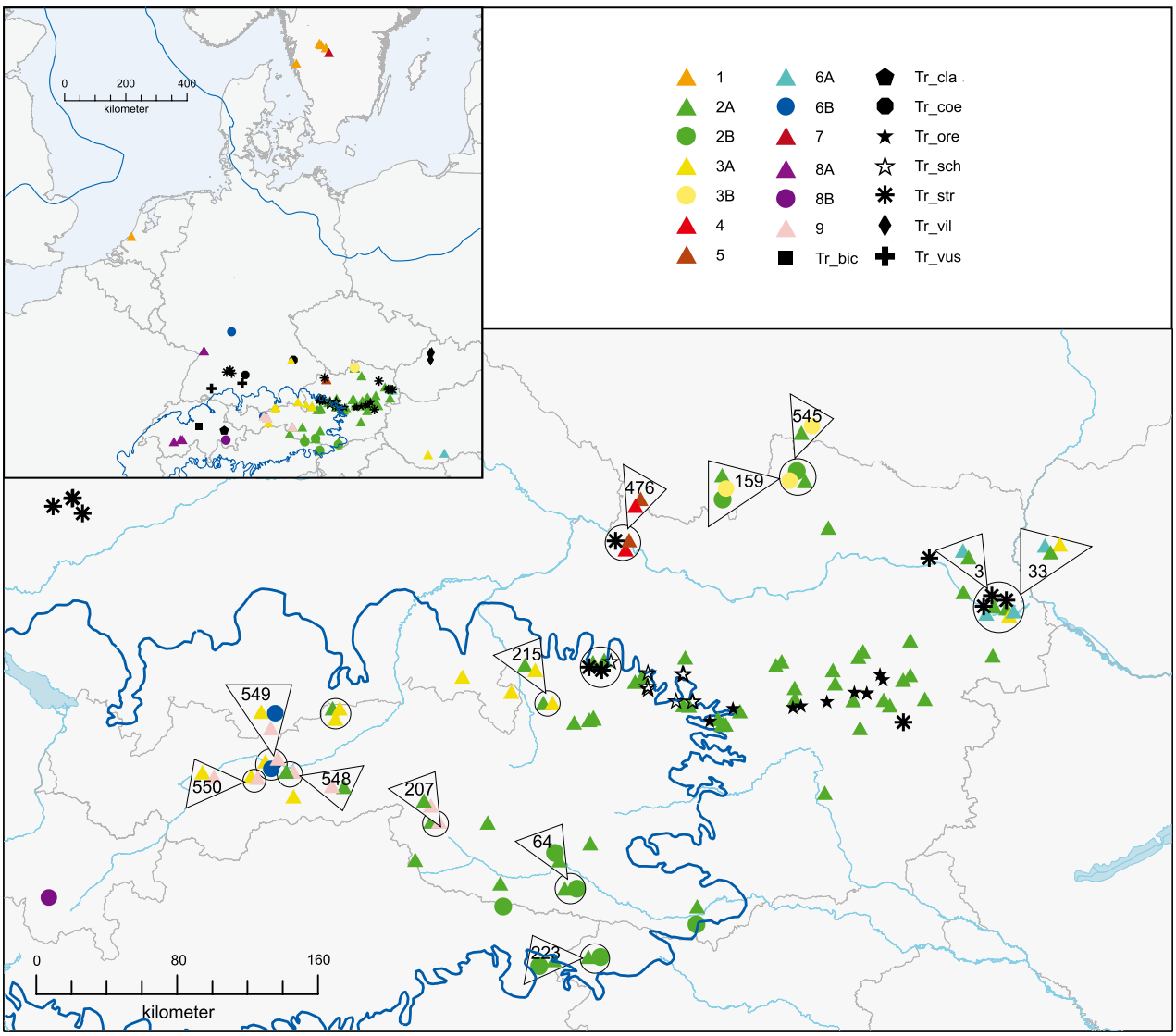

Fig. 1 Sampling sites and distribution of clades. Circles indicate regions in which the sampling sites were too dense to be depicted with their actual distances and therefore had been manually decompressed. Triangles indicate sampling sites at which several clades co-occur. The dark blue lines indicate the maximum extent of glaciers (35–19 ka ago) during the Würm ice age. Abbreviations: Tr_bic: *Trochulus biconicus*; Tr_cla: *Trochulus clandestinus*; Tr_coe: *Trochulus coelomphala*; Tr_ore: *T. o. oreinos*; Tr_sch: *T. o. scheerpeltzi*; Tr_str: *Trochulus striolatus*; Tr_vil: *Trochulus villosulus*; Tr_vus: *Trochulus villosus*

Trochulus villosulus (Rossmässler 1838) and *Trochulus striolatus* (Pfeiffer, 1828) were determined using morphological traits described in the literature (e.g. Ložek 1956; Kerney *et al.* 1983; Proćków 2009). *Trochulus oreinos oreinos* and *Trochulus oreinos scheerpeltzi* were assigned using the original description (Wagner 1915; Mikula 1957) as well as by comparisons with reference specimens (paratypes) from the collections of the Natural History Museum Vienna (NHMW). Information on individuals and localities is compiled in Table S1.

Living specimens were drowned in the laboratory, and DNA was extracted according to the protocol of Kruckenhauser *et al.* (2011). If available, DNA was extracted from three adult individuals of each locality. In total, 389 individuals were used for this analysis, among them three *T. biconicus*, eight *T. villosus*, four *T. coelomphala*, three *T. clandestinus*, six *T. villosulus*, 39 *T. striolatus*, 32 *T. o. oreinos* and 28 *T. o. scheerpeltzi*. Twenty-six individuals of *T. o. oreinos* and 27 of *T. o. scheerpeltzi* have also been used in earlier studies (Duda *et al.* 2010, 2011). The remaining 261 individuals were morphologically assigned to *Trochulus hispidus* or tentatively to *Trochulus sericeus*; 79 of them were already included in earlier studies of Duda *et al.* (2010, 2011). Six individuals representing out-group taxa were used: three individuals of *Isognomostoma isognomostomos* (Schröter, 1784), one of *Monacha cantiana* (Montagu, 1803) and two of *Plicuteria lubomirski* (Ślósarski, 1881).

Genetic analysis

From all individuals, a partial region of the mt *cytochrome c oxidase subunit I* (*COI*) gene was analysed. In addition, from representatives of each clade, partial regions of the mt *16S rRNA* (*16S*) and the *12S rRNA* (*12S*) genes were also sequenced (98 individuals). As out-group taxa, *Monacha cantiana* (one specimen) and *Plicuteria lubomirski* (two specimens) were analysed with all three markers. Primer binding sites correspond to those used by Gittenberger *et al.* (2004) for *COI* and by Pfenninger *et al.* (2003) for *16S*. Primers were optimised based on the alignments of several snail species and published in Duda *et al.* (2011). Primer sequences for the *12S* fragment were designed by Cadáhia *et al.* (2013): 12SGast_fwd2 5′-AGTGACGGGC GATTTGT-3′, 12SGast_rev3 5′-TAAGCTGTTGGGC TCATAAC-3′. Resulting fragment sizes (including primers) were 705 bp (*COI*), 391–399 bp (*16S*) and 689–703 bp (*12S*).

As a nuclear (nc) marker, we analysed a region including partial sequences of two rRNA genes and the spacer region in between (*5.8S–ITS2–28S*) using two overlapping fragments generated with the two primer combinations: 5.8S_LSU-1fw 5′-CTAGCTGCGAGAATTAATGTGA-3′, 28S_LSU-2fw 5′-GGGTTGTTTGGGAATGCAGC-3′ and 28S_LSU-3rv 5′-ACTTTCCCTCACGGTACTTG-3′, 28S_LSU-4rv 5′-GTTAGACTCCTTGGTCCGTG-3′ (all from Wade & Mordan 2000). The combined alignments resulted in a sequence of ~1360 bp. PCR was performed on a Master Gradient thermocycler (Eppendorf) in 50 μl with 1 unit Taq DNA polymerase (Roche), 1 μM 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*, both nuclear fragments) and 55 °C (*16S* and *12S*). 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 by direct sequencing (both directions). Within the PCR fragment 5.8S_LSU-1fw/28S_LSU-3rv, six heterozygous individuals with length polymorphisms were detected, and hence, their sequence could not be determined by direct sequencing. The corresponding PCR fragments were cloned: PCR products were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Düsseldorf, Germany) and cloned (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA); four clones per individual were sequenced. Sequencing of both strands was performed by LGC Genomics (Berlin, Germany) using the original PCR primers or (for cloned PCR products) M13 universal primers.

Data analysis

Sequences were edited in BioEdit version 5.0.9 (Hall 1999). This software was also used to translate the DNA sequences of the *COI* into amino acid sequences using the invertebrate mitochondrial code. For the *COI* sequences, the alignment was straightforward because there were no insertions or deletions. Alignments of the mt sequences *16S* and *12S* as well as for the nc sequences (*5.8S–ITS2–28S*) were performed with ClustalX 2.0.12 (Larkin *et al.* 2007) using the default parameters. Lengths of the alignments were 377 bp (*16S*), 699 bp (*12S*) and 1364 bp (*5.8S–ITS2–28S*). The *16S* and the *12S* alignments were trimmed using the automated option in trimAl *v.3.1* (Capella-Gutierrez *et al.* 2009) at the Phylemon 2 server (Sánchez *et al.* 2011), resulting in a 351-bp alignment for the *16S* and a 654-bp alignment for the *12S* sequences, and these trimmed alignments were used for all further analysis.

A test for substitution saturation (Xia *et al.* 2003) was performed with DAMBE 5.2.68 (Xia & Xie 2001) for the complete *COI* alignment, as well as for all single codon positions separately. Additionally, using the DAMBE graphic tool, transitions were plotted against transversions to obtain a graphic representation of the saturation. The results of the saturation test suggested using all characters, although the third codon position showed a moderate degree of saturation in the plots.

Average p-distances (pairwise exclusion of gaps) were calculated using *MEGA* version 4 (Tamura *et al.* 2007), which was also used to calculate neighbour-joining (NJ) trees (Saitou & Nei 1987). For the NJ tree of the nc sequences, we used mid-point rooting. Nodal support was evaluated with non-parametric bootstrapping based on 1000 replicates. A search for the best-fitting substitution model was performed using the Akaike information criterion corrected for small sample size (AICc) as implemented in the jModeltest 0.1.1. (Posada 2008).

Bayesian analyses (BI) were performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001), applying the models of sequence evolution for nucleotide sequences as suggested from jModeltest (HKY + I + G for the three partitions: *COI*, *16S* and *12S*). Runs were started with random trees and performed for 3 million generations each with four Markov chains and a sampling frequency of every 100th generation. Those trees generated prior to the stationarity were discarded as burn-in and were not included in the calculation of the consensus trees.

A full median-joining (MJ) network (Bandelt *et al.* 1999) was constructed with Network 4.6.0.0 (available at www.fluxus-engineering.com), putting equal weight on each site and using the postprocessing option 'mp calculation' for the following data sets: *COI* of clade 2A, *COI* of *T. striolatus* as well as for the nuclear sequences. The number of haplotypes was determined with ARLEQUIN 3.11 (Excoffier *et al.* 2005).

The sequences determined in this study are deposited at GenBank under the accession numbers *COI*: KJ151294 - KJ151548, *16S*: KJ151549 - KJ151617, *12S*: KJ151618 - KJ151718, *5.8S*-ITS2-*28S*: KJ151719 - KJ151767. The material is deposited in the mollusc collection of the Natural History Museum Vienna (Mollusca NHMW 109000/AL, individual Ids see Table S1).

Results

Variation in COI sequences

The analysis of the partial mt *COI* gene (660 bp) amplified from 389 individuals revealed 203 different haplotypes. In the deduced amino acid sequence, most (274 of 383) individuals are identical, with a maximum number of five replacements recorded between two individuals. The most common amino acid sequence occurs in specimens from six different clades and in three species, but there is variation within clades and species (data not shown).

The sampling area (altogether 126 localities) is displayed in Fig. 1. Fig. S1 shows the NJ tree of the *COI* DNA sequences with several quite distinct and highly supported clades. Two clades (4 and 7) are represented by only one individual each; the others constitute groups of up to 158 (clade 2A) individuals. The clades obtained maximum support, with the exception of clade 3A. The relationships between the clades, however, are not well resolved. Specifically, the more basal nodes are poorly supported.

Seven clades represent taxa that are clearly classified according to morphological features: the species *T. villosus*, *T. clandestinus*, *T. villosulus*, *T. striolatus*, *T. biconicus*, as well as the two subspecies of *T. oreinos* (*T. o. oreinos* and *T. o. scheerpeltzi*). Another clade was tentatively assigned to the species *T. coelomphala*, although this assignment is ambiguous because one of the four individuals in this clade is morphologically very similar to *T. hispidus*. Besides these taxa, the nine remaining mtDNA clades (1–9) comprise individuals that displayed a high variation in shell size and morphology and were mainly assigned to *T. hispidus*, while some displayed a more *T. sericeus*-like phenotype. Morphological variation in each clade was high, and none of the clades was composed of *T. sericeus*-like phenotypes exclusively. In the following, we refer to the nine clades as '*T. hispidus* complex' (for details of the morphometric study and the taxon *T. sericeus*, see Duda *et al.* revised). Four of these clades are further subdivided into subclades (Fig. S1). The definition of clades and subclades was based on the criteria: high branch support (above 95%), limiting the maximal p-distance within a clade (7%). Interspecific distances and distances within clades are presented in Table S2.

Partial trees showing all individuals of each clade or subclade are presented in figures (Figs S2–S4) and described in detail below.

Phylogenetic relationships between mtDNA clades

Two additional mtDNA marker sequences (*12S* and *16S*), which in general contain conserved parts with lower substitution rates, were sequenced from representatives of each clade (101 individuals). In the BI tree calculated from the concatenated mtDNA sequences (*12S*, *16S* and *COI*; altogether 1660 bp; Fig. 2), most nodes are well supported, and only two have posterior probabilities (pp) of less than 95%. Hence, concerning the phylogenetic relationships between the clades, we will refer only to the concatenated tree.

In this tree, the basal node separates *T. oreinos* with its two monophyletic subspecies, and the next node separates *T. biconicus* from a paraphyletic group comprising the *T. hispidus* complex and morphologically well-separated species. This paraphyly is supported with high confidence. This paraphyletic assemblage contains two main groups: one is formed by *T. villosus*, *T. clandestinus* and clade 8 of the *T. hispidus* complex, the latter being the sister group of *T. villosus* and the second main group contains (i) clade 6, (ii) the highly supported sister clades 1 and 9 and (iii) a group with the two sister species *T. striolatus* and *T. villosulus* as well as the remaining clades of the *T. hispidus* complex (2, 3, 4, 5, 7) together with *T. coelomphala*.

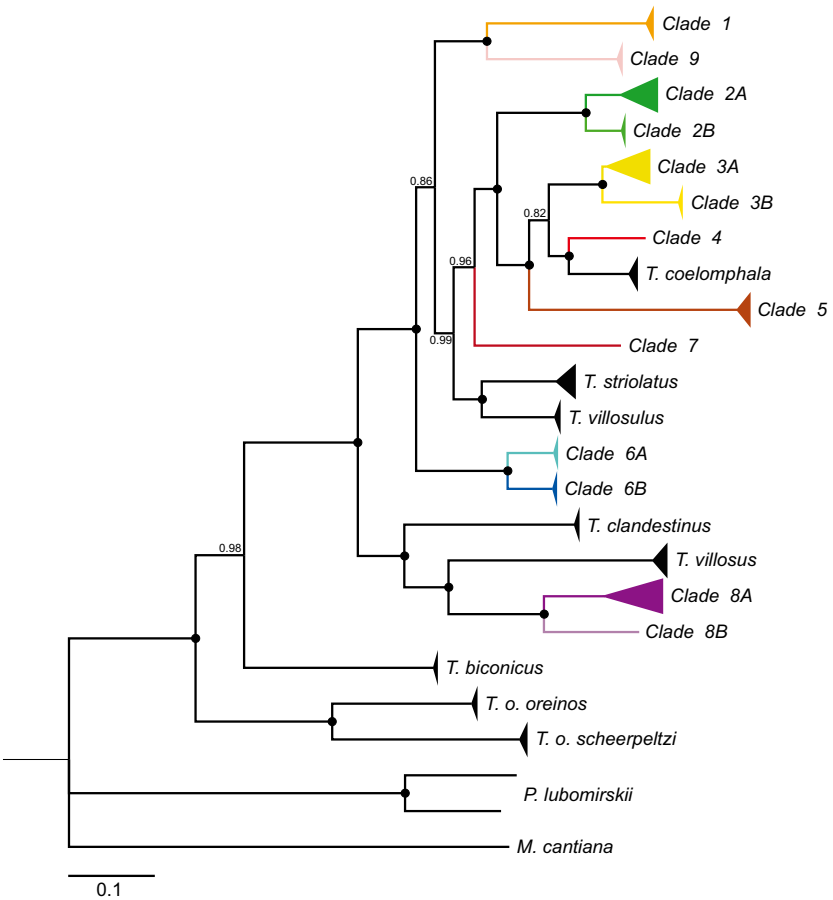

Fig. 2 Bayesian tree of the concatenated *COI*, *16S* and *12S* sequences. Posterior probabilities are given for all nodes. Black dots indicate maximum support. The scale bar indicates the expected number of substitutions per site according to the model of sequence evolution applied. The colour code is the same as in Fig. 1.

Variation and geographic distribution of the mtDNA clades of the* T. hispidus *complex

Distances between clades exceed in several cases those between undisputed species; this is evident in Table S2, which shows the uncorrected mean p-distances of the *COI* sequences. Distances within clades and subclades ranged up to 6.7% (Table S2) and are illustrated in partial trees (Figs S2–S4). Concerning the distribution of the clades of the *T. hispidus* complex, some geographic patterns become apparent (Fig. 1). Clade 1 represents the western and northernmost localities with specimens from Sweden, which is the type locality of *T. hispidus*, and from the Netherlands. Clade 2 is further divided into two subclades: subclade 2A represents by far the largest sample. It is widely distributed in Austria from the eastern part of the Austrian Alps and adjacent hills and flatland areas up to Tyrol in the west. In the east, it was found in localities along the Danube River, and in the south, it was found close to the Italian and Slovenian borders. In Central Austria exclusively this subclade was found. Subclade 2B shows a disjunct distribution in the north and south of Austria, at the margins of the area covered by subclade 2A. Clade 3 is also divided into two subclades: 3A has a disjunct distribution and shows quite high distances within clades (up to 6.4%). In the west, it was found in mountainous regions of western Austria (Tyrol, Salzburg) and along the Danube in southern Germany (Bavaria); in the south-east, it was detected in the Mecsek Mountains in southern Hungary. Unlike in clade 2A, the individuals in clade 3A cluster according to geographic areas. Subclade 3B was found at only one locality in northern Austria, in the Waldviertel region of Lower Austria. Clade 4 is represented by a single individual, which was found close to the Danube River in Upper Austria, the same locality in which the two individuals forming Clade 5 were also collected. Clade 6 seems to be restricted to the Danube and some of its larger tributaries: subclade 6A, which shows quite low within-clade distances, is restricted to the Danube River floodplains (it was found in localities close to Vienna and in southern Hungary), while 6B occurs along the Inn River in western Austria and the Tauber River in Baden-Württemberg (Germany). Clade 7 consists of a single individual from Sweden, which was found at a locality close to that of clade 1. Subclade 8A was detected in south-western Switzerland

and at the Rhine River (Baden-Württemberg, Germany), and subclade 8B consists of two individuals from one locality in eastern Switzerland. Finally, Clade 9 was found at three localities in Tyrol (Austria) and shows quite low within-group distances.

In summary, nine of the 13 clades and subclades described in the present study occur also in Austria. Furthermore, several quite distinct clades co-occur at the same localities. Nonetheless, some of the clades apparently have disjunct distributions, and in some cases, very similar sequences were found in quite distant localities. Although only few individuals were analysed at most of the 126 sampling sites, at 12 sampling sites, individuals from different (up two-three) mtDNA clades or subclades were obtained (see triangles in Fig. 1). All but one of those 12 sampling sites are located at the margins of the Austrian sampling area. In the central region, there is a wide area in which only subclade 2A was recorded. The following clades occurred together: 2A + 2B, 2A + 9, 3A + 9, 3A + 6B + 9, 4 + 5, 2A + 2B + 3B, 2A + 3B, 2A + 6A, 2A + 3A + 6A.

To assess possible geographic structures in more detail, *COI* networks were calculated for the subclades 2A and 3A, which include sufficient numbers of individuals (158 and 33, respectively). Fig. 3 shows a median-joining network of subclade 2A, where the colours represent eight geographic regions. The 72 haplotypes form five haplogroups, but none of them has a central position. Group 1 consists almost exclusively of individuals from the north-eastern Alps. However, many individuals from that region occur also in three other clades (2, 3 and 4). Groups 2–5 cannot be assigned to a specific geographic region. Individuals from each of the other seven regions are found within two to four haplogroups. Altogether, no clear geographic clustering of haplotypes is evident within subclade 2A. The network of clade 3A (data not shown) consists of 20 haplotypes arranged in a geographic pattern. The three individuals from the westernmost locality, 'Stubaier Alpen' (spID 244), have different haplotypes, which are all in the centre of the network. They are separated by only few (up to six) substitutions from most of the other south-western individuals in this clade. In contrast, the individuals from the eastern and northern localities have quite distinct haplotypes (18–23 substitutions), which are well separated from each other. Only the individuals from the 'Berchtesgadener Land' (spID 407 and 412) are present in two different groups: one closer to the western localities (spID 407 with eight substitutions) and the other very distantly related (spID 412 with 18 substitutions).

Genetic variation within T. striolatus *and* T. oreinos

Concerning the other species, which form well-supported monophyletic groups in the trees, the data allow meaningful considerations about the genetic variability regarding *T. striolatus* and *T. oreinos*. Besides *T. hispidus*, only these two species were sampled over a broader geographic range. For *T. striolatus*, our sample covers a wide geographic area ranging from Baden-Württemberg (Germany) to Lower Austria. The genetic variability is, compared with that found in the *T. hispidus* complex, low (maximum p-distance 3.7%, Table S2). The three different subspecies of *T. striolatus* (*striolatus*, *juvavensis* and *danubialis*) are not clearly separated on the basis of the *COI* mtDNA data (Fig. S3). The individuals are clustered in five haplogroups (Fig. S3) reflecting their geographic origins. Two haplogroups consist exclusively of individuals from Germany (hg1 and hg2), whereas the three remaining haplogroups comprise the samples from Austria. Among the latter, one is formed by individuals found along the Danube River (hg3), one is formed by individuals from the Upper Austrian Höllengebirge (hg4), and a closely related one (hg5) includes individuals from both regions. In the MJ network (data not shown), this haplogroup (hg5) has a central position.

For *T. oreinos*, the investigated areas cover the entire distribution areas of both subspecies, and most of them were already included in the study by Duda *et al.* (2011). The genetic variability within each of the *T. oreinos* subspecies is rather low (Table S2) with no clear geographic pattern.

Nuclear marker sequence

The results of the mtDNA analysis raised the question whether the various clades of the *T. hispidus* complex belong to a single species or might in fact represent several cryptic species. To further approach this question, we analysed a nc marker sequence (*5.8S–ITS2–28S*) from a subset of individuals representing all clades (except clade 9) and all other species included in the concatenated tree (except the outgroup species).

From six individuals, more than one sequence was obtained by sequencing several clones. At most, three different copies differing by up to five substitutions were found within individuals. The overall mean p-distance for the *ITS2* region was 1.7%, while for the *28S* region, it was 0.8%. The NJ tree based on these sequences (Fig. 4) reveals a very low differentiation of most of the clades found in the mtDNA tree. However, there are some exceptions: *T. oreinos* is clearly separated, like in the mtDNA trees. Also, its two subspecies are well differentiated. Furthermore, *T. clandestinus* forms a separate branch. The remaining sequences are present in one clade without any clear pattern. They represent individuals from all analysed clades of the *T. hispidus* complex as well as members of the species *T. villosus*, *T. villosulus*, *T. striolatus* and *T. coelomphala*.

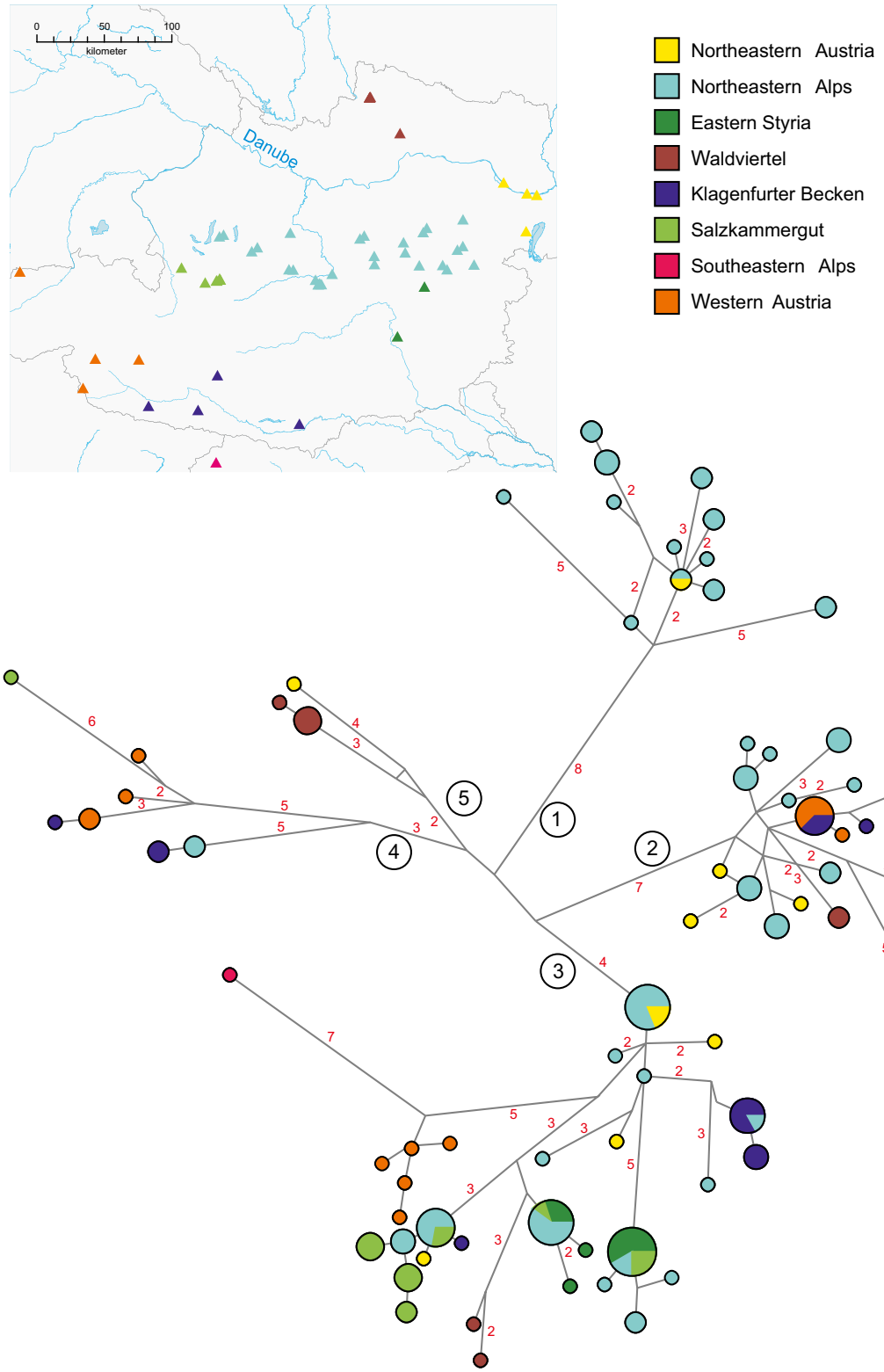

Fig. 3 Median-joining network of the *COI* sequences from subclade 2A. The branches are not drawn to scale, but the number of substitutions is given in red numbers. The size of the circles corresponds to the number of individuals possessing the same haplotype. The geographic origin is reflected by the colours as shown in the map. The five main haplogroups are numbered.

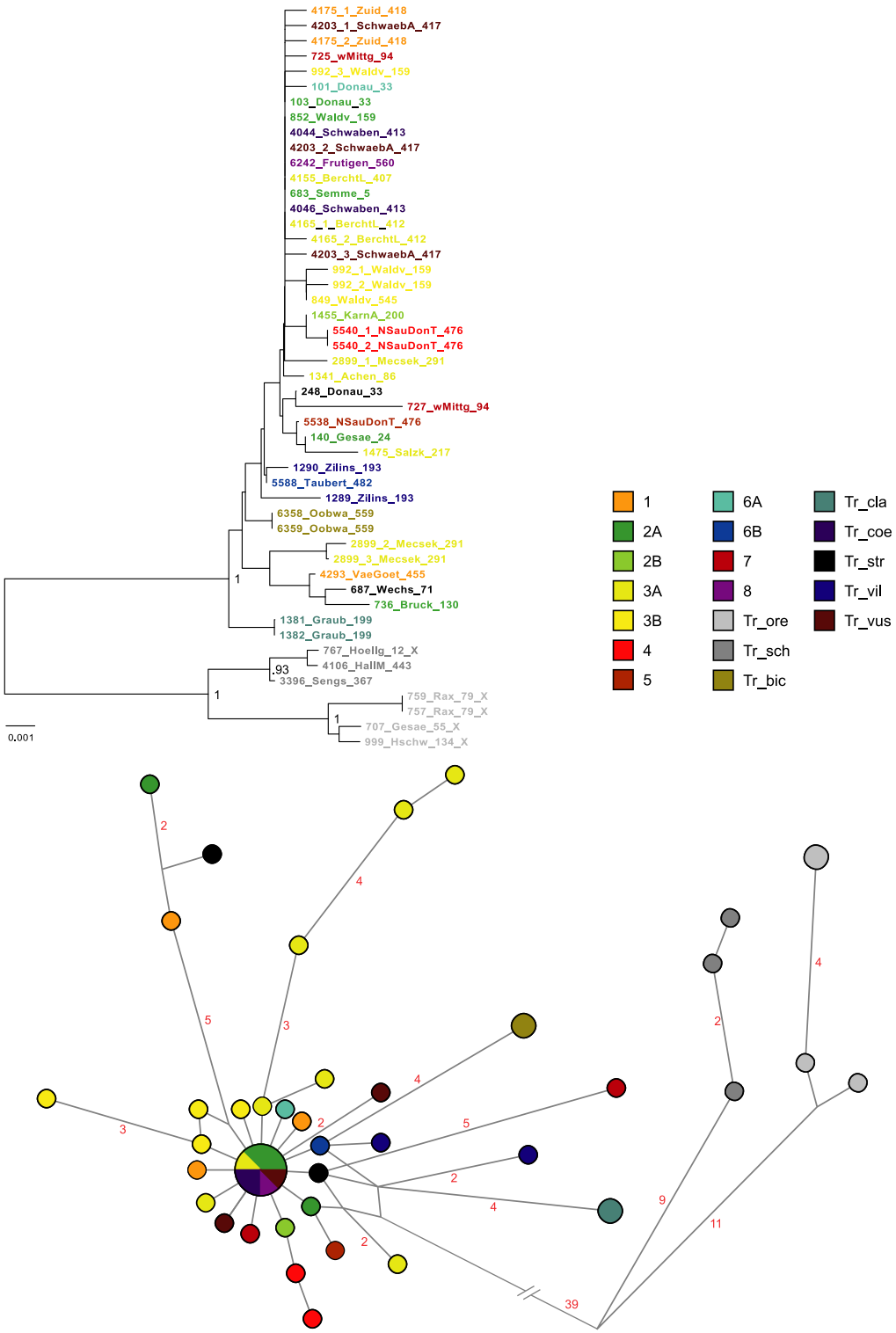

Fig. 4 Neighbour-joining tree and median-joining network of the nuclear *5.8S–ITS2–28S* sequences. In the median-joining network, the branches are not drawn to scale, but the number of substitutions is given in red numbers. The size of the circles corresponds to the number of sequences possessing the same haplotype. Abbreviations are the same as in Fig. 1.

Most sequences were identical, while 12 sequences differ from this most frequent haplotype by only one substitution. The remaining sequences differ at up to nine positions (substitutions and indels). The same data set was used to calculate a network (Fig. 4), which illustrates the lack of differentiation between most of the mtDNA clades.

Discussion

Paraphyly of T. hispidus

The most prominent outcome of this study is the existence of nine highly distinct mtDNA clades, which all are composed of snails with either a *T. hispidus* or a *T. sericeus* morphology. These clades form a paraphyletic assemblage as they are intermingled with clades representing morphologically clearly defined species (e.g. *T. villosus*, *T. villosulus* and *T. striolatus*), each of them forming a well-supported clade. At the current state of knowledge we have no reason to question their species status. Their clear morphological and anatomically differentiation can be interpreted as a reflection of the overall separation of the nc genomes, although this separation is not found in the ncDNA tree. There are several explanations for the incongruence between mtDNA and ncDNA data: incomplete lineage sorting, ongoing or at least recent gene flow among these taxa or a combination of the two possibilities, with only sporadic gene flow, which is, however, sufficient to slow down the process of lineage sorting. The fact that the *T. hispidus* complex appears paraphyletic in our mtDNA tree could be explained with (i) budding speciation (see Fig. 1k in Funk & Omland 2003), which has been found in other organism groups (Vanderpoorten & Long 2006; Toussaint *et al.* 2013). In this scenario, the emerging daughter species leave behind the parental species paraphyletic until lineage sorting is completed. The daughter species are expected to be monophyletic, and in theory, parallel patterns in ncDNA and mtDNA data should be found (Funk & Omland 2003), which is not the case with our data set. One example of land snails has been reported for the Cretan genus *Xerocrassa*, in which the mtDNA tree revealed several species as paraphyletic (Sauer & Hausdorf 2009). Yet in an extensive AFLP analysis, most of those species were monophyletic (Sauer & Hausdorf 2010). (ii) Alternatively, the paraphyly might be due to past introgression of the mt genome from *T. hispidus* into other species and subsequent divergence in mt lineages. (iii) Finally, some of the mt clades may actually represent yet unknown cryptic species, while others may not. If clades 8A, 8B, 6A, 6B, 1 and 9 (*T. coelomphala* is not considered because of insufficient sampling) proved to be cryptic species, the paraphyly of *T. hispidus* would be abolished.

The genetic distances between the mtDNA clades of the *T. hispidus* complex are extremely high, often exceeding those between morphologically well-defined species. For example, *T. striolatus* and *T. villosulus*, which are sister species in the mtDNA tree, are separated by a p-distance of 9.5% (*COI*), while the range of mean distances between the clades of the *T. hispidus* complex is between 10.7 and 18.9%. A similar result – the presence of highly divergent clades – was obtained by Pfenninger *et al.* (2005), who investigated mainly individuals from German, Swiss and French sample sites. From the six clades attributed to the *T. hispidus/sericeus* complex by Pfenninger *et al.* (2005), only two are closely related to clades detected in the present study: clade 7 (represented by only one individual from Sweden), which has about 3% distance to lineage A within the *striolatus/plebeius* clade of Pfenninger *et al.* (2005), and clade 8A. Thus, altogether, at least 14 clades are currently known (subclades not counted), but it is evident that the definition of clades/subclades is somewhat arbitrary and differs between studies. Nonetheless, many clades probably still remain undetected. Specifically, the sampling at the western and eastern margins of the distribution is not yet sufficient. A final picture will become available only when the sampling grid covers the entire distribution range.

Species delimitation in the T. hispidus *complex*

In general, the variation in shell morphology and size is high in all clades of the *T. hispidus* complex, and most clades comprise individuals with 'typical' *T. hispidus* habitus as well as individuals displaying the *sericeus* type (narrow umbilicus, globular shell). This was confirmed in a parallel study of morphological and anatomical characters (Duda *et al.* revised), where the *T. sericeus* phenotype could not be exclusively assigned to a specific clade. However, the specimens analysed here might be specifically distinct from the true *T. sericeus*, and this species has not been covered by our sampling. Therefore, especially investigations of samples from France, which is the type locality for *T. sericeus*, would be very elusive.

There was no clade comprising all individuals with a *T. sericeus* phenotype, but also none of the nine clades representing the *T. hispidus* complex was differentiated in the shell morphological or anatomical analyses (Duda *et al.* revised). Moreover, the mtDNA clades were not recovered in the ncDNA tree.

The high evolutionary divergence in the mt markers could be accompanied by genomic incompatibility preventing gene flow between co-occurring clades. This was described for the marine copepod *Tigriopus californicus* (Rawson & Burton 2002; Willett 2006). Yet, the number of amino acid replacements in the *COI* sequence within all *Trochulus* clades is rather limited, and they do not correspond to the mt clades. Hence, genomic incompatibility due to the high mitochondrial divergence probably had no major impact in *Trochulus*.

The question whether some of the clades might represent cryptic species is difficult to address on the basis of the current knowledge. Considering the lack of knowledge about the reasons for the incongruent results of the mtDNA and the ncDNA sequences, it appears not meaningful to apply species delimitation methods as, for example, in Prévot *et al.* (2013). Elevating clades to species status to eliminate the paraphyly of *T. hispidus* solely to force taxonomy to reflect the gene tree is not reasonable (Funk & Omland 2003; Zachos 2009; Zachos *et al.* 2013). Although this would be in accordance with the phylogenetic species concept (e.g. Cracraft 1983; Nixon & Wheeler 1992), the fact that *T. hispidus* appears paraphyletic in our mtDNA tree is in our opinion not a sufficient argument for splitting it into different species. There are already several names available for shell variants in the *T. hispidus* complex, which are currently considered as synonyms; therefore, the identity of already existing names has to be clarified before any other nomenclatural consequences are drawn.

Concerning species delimitation, a straightforward approach following the biological species concept (Mayr 1942, 1970) is to test gene flow between co-occurring clades. Such analyses were conducted by Depraz *et al.* (2009) for the Swiss endemic species *T. piccardi* and the partially co-occurring *T. hispidus/sericeus* lineage F. According to that study, restricted gene flow (assessed by microsatellite analysis and mt sequences) indicated that, despite rather small mtDNA divergence, the two lineages are actually distinct species. This finding corroborates earlier morphological analyses by Pfenninger & Pfenninger (2005). In a tree combining the *COI* data of the present investigation with those of Depraz *et al.* (2009), *T. piccardi* is nested within our clade 8A, where it is most closely related to four Swiss individuals of our study (data not shown). This might be interpreted as a hint that more cryptic species might be hidden among the clades of the *T. hispidus* complex.

In our sample, there are several localities where representatives of two or even three clades occur together (black-lined triangles in Fig. 1), and it is likely that there are many more regions where the distribution of clades overlaps. During our sampling, we did not observe that specimens from different clades, which actually occur syntopically, have any recognisable difference in their local environment from which they were taken (e.g. plants). However, we are aware that representatives of these clades might be differentiated in biological parameters not investigated a so far. Most of the localities harbouring different clades are situated around the distribution range of subclade 2A. Such localities are ideal settings to test whether or to which extent there is gene flow between individuals belonging to different mtDNA clades. Sympatry of distinct clades without gene flow would be evidence for the presence of distinct species, which might be differentiated by biochemical or life history traits. Currently, we are planning further investigations of the *T. hispidus* complex with an explicit focus on species delimitation which will be a prerequisite for taxonomic long-term decisions. Concerning the conservation issue, we have to mention that several clades within the *T. hispidus* complex and local populations of *T. striolatus* are under pressure, and this should be taken into consideration (for details see Duda *et al.* revised).

Age of the clades

As mentioned above, the existence of highly divergent mtDNA clades suggests that the radiation of the *T. hispidus* complex started long before the Pleistocene. The earliest fossil record in Central Europe is from the early Pleistocene (according to Frank 2006; Ložek 1964). Still, the fossil record for the Pliocene and the early Pleistocene is in general scarce, and the assignment of fossils is very problematic due to small conchological differences. Therefore, a reliable calibration to date the splits in our tree is hardly possible, and we refrained from performing a molecular clock analysis. The high mtDNA distances between clades could also be due to an accelerated mutation rate as it was suggested for other snails (e.g. Thomaz *et al.* 1996). In our ncDNA data set, only the two subspecies of *T. oreinos* form clear clades in both the mt and nc trees. They are separated by 0.9% mean p-distance in the ncDNA and by a mean distance of 13.7% (15 times higher) in mtDNA. This is in the same order of magnitude as the relation between mt and nc rates reported for other organism groups (e.g. 10 times for mammals; Li & Graur 1991). Thus, as our data do not allow to propose an accelerated mt substitution rate, one might ask for explanations for the persistence of these highly divergent clades over a presumably very long time. One possibility is that they (or some of them) might represent cryptic species. This possibility will be tested in a forthcoming study. Alternatively, what we observe as the *T. hispidus* complex might be indeed a paraphyletic species that retained very large population sizes over long periods of time. From population genetic theory (Avise 2000), monophyly is expected for neutral markers after $4N_e$ generations (N_e being the effective population size). In this context, it has to be considered that N_e in hermaphrodites depends on whether or not they are simultaneously hermaphrodite, to what extent they may self-fertilise and to what extent they show multiple paternities. A large N_e has been reported in, for example, *Cepaea* (Murray 1964), but for *T. hispidus*, no data are available, although large population sizes are probable for this widely distributed species, which frequently has connected habitats along rivulets and

rivers (Duda *et al.* 2010). Under this hypothesis, however, it is surprising that the haplotypes are arranged in quite separated clades with a geographic pattern rather than a bush-like bundle of haplotypes. This could be explained by the survival and divergence of the highly distant mtDNA clades in isolation over long periods, especially in the cold phases of the Pleistocene. This is supported by ample fossil record of *T. hispidus* throughout the Pleistocene, which shows a wide distribution, as indicated, for example, by findings in France, Austria, Hungary, Serbia, Croatia and the Czech Republic exist (Binder 1977; Frank *et al.* 2011; Ložek 1964; Marković *et al.* 2005; Molnár *et al.* 2010; Rousseau *et al.* 1992; Sümegi *et al.* 2011). The scenario of long-lasting isolation does, of course, not exclude the possibility of sporadic contact with gene flow. At least for several clades, the present co-occurence is evident from our data. High intraspecific mtDNA variability which was explained by refugial isolation and secondary contact has been found also in other land snail species, for example, in *Arianta arbustorum* (Haase *et al.* 2003, 2013).

Phylogeographic considerations

There is a geographic pattern in the comprehensive genetic tree (Fig. 2). This might reflect the phylogeographic history of the taxa investigated. Besides *T. biconicus* and *T. oreinos*, which split from the basal nodes, the main group in the tree, which contains the *T. hispidus* complex, is further subdivided into two clades: (i) one with a more eastern/northern distribution comprising clades 1–7 and 9, *T. coelomphala*, *T. villosulus* and *T. striolatus* and (ii) a western one consisting of clade 8, *T. clandestinus* and *T. villosus*. A similar separation can also be found in Pfenninger *et al.* (2005), which described a group with more western clades (*T. piccardi*, D, E, F, G, H and I) and a group with predominantly eastern clades (A, B and C). This suggests two old radiations starting from unknown western, respectively, eastern regions. The distant position of *T. biconicus* and *T. oreinos* reinforces further investigations on other related taxa, for example, endemics with small distribution ranges such as *T. montanus* and *T. caelatus* or even the species of the related genus *Petasina*, which are integrated into *Trochulus* by some authors such as Proćków (2009) and Welter-Schultes (2012).

Concerning glacial refugia, the data still have to be considered as preliminary. For most clades of the *T. hispidus* complex, the present distribution is not assessed yet, and thus, considerations where potential refugia might have been located remain speculative. Anyhow, the *T. hispidus* complex appears to have spent the glacial periods in several different refugia.

The distribution of clade 2A (Fig. 1) suggests that it persisted even throughout glacial periods in the area east of the ice sheet. This interpretation is supported by its exclusive occurrence in those areas, where it does not coexist with any other clade. Other occurrences of 2A in the far west and east of Austria may be the result of postglacial west- and eastward immigration (Fig. 1). The high variation within-clade 2A (up to 4.4%), even within the formerly glaciated region, implies that the re-colonisation started from a large area, in which the variability had not been drastically reduced by a bottleneck. The ample existence of *T. hispidus* in the fossil record of the Pleistocene loess deposits from Lower and Upper Austria, which also includes the cold phases (Binder 1977; Frank *et al.* 2011) corroborates the hypothesis that this area was a suitable habitat for *T. hispidus* even throughout the glacial periods.

Clade 2B shows a disjunct distribution with a few occurrences in northern Austria as well as in the south-eastern Alps. This finding is interesting given the low genetic distance between the southern and eastern sample. So far, it is unknown whether this clade has a wider distribution.

For areas in western Austria covered by glaciers during the Last Glacial Maximum, a postglacial re-colonisation must be assumed. The occurrence of five quite differentiated clades within this region suggests that they immigrated from different refugia. As *T. hispidus* is apparently an euryoecious species (Proćków *et al.* 2013), dispersal could have taken place quite fast. The locations of those presumed refugia – except of clade 2A, for which a colonisation from the east is plausible – remain speculative. The same applies to the clades found in Sweden, the type locality of *T. hispidus*, which was covered by an ice shield during the last glacial. In our study, we detected two highly distinct lineages there.

Despite the fact that *T. striolatus* is an euryoecious species like *T. hispidus* (Kerney *et al.* 1983; Proćków 2009; Duda *et al.* 2010), it shows quite low intraspecific variation in the mtDNA (maximum 3.7%) and slightly smaller shell morphological variability (Duda *et al.* revised). The three subspecies investigated (*striolatus*, *juvavensis* and *danubialis*) are not clearly separated in the *COI* tree (Fig. S3); there is, however, a geographic pattern indicating that the populations of *T. striolatus* investigated in this study may have been distributed over a wide range during the last glacial. Given the limited sampling in the present study, final conclusions about the phylogeography of this species should include samples from the whole distribution area.

We assume a completely different situation for the *T. oreinos* taxa. Their potential dispersal ability is quite poor due to their specific ecological niche of patchy caricetum-firmae meadows and cool alpine rock areas nearly free of vegetation (Duda *et al.* 2010). As most parts of their current distribution area remained ice-free during the Last

Glacial Maximum (Van Husen 1997), we conclude that they survived at least the last glaciation within this region at the north-eastern margin of the Eastern Alps. Alpine glacial refugia in the same regions were proposed earlier (Schönswetter *et al.* 2002; Rabitsch *et al.* 2009). The ranges of the two subspecies reflect two separated glacial refugia. Similar cases are documented for several endemic invertebrates and vascular plants in the north-eastern Alps (Rabitsch *et al.* 2009).

Consequences for DNA barcoding

Not all species of the genus *Trochulus* can be easily recognised morphologically; some are distinguished solely by non-distinct characters (e.g. wider umbilicus) and others by anatomical features (Schileyko 1978; Proćków 2009). Moreover, juveniles are hardly determinable, and hence, a DNA barcoding approach could be helpful for identifying individuals collected in the field (e.g. for a biodiversity inventory). Nonetheless, as evident from the data – at least at the current state of knowledge – any barcoding attempt concerning the *T. hispidus* complex appears futile. If a species is not monophyletic in the mtDNA gene tree, it cannot be assigned with a *COI* barcode, even if the thresholds are set very high. Before species delimitation in the *T. hispidus* complex is accomplished on the basis of more data, trying to define a DNA barcode for *T. hispidus* does not make sense. Difficulties in molecular species delimitation on the basis of DNA data, and hence also for DNA barcoding, have been reported frequently for snails (Davison *et al.* 2009; Sauer & Hausdorf 2012) reflecting the problems of cryptic species on the one hand and high mtDNA divergence within species on the other hand.

This problem is less relevant for the other *Trochulus* species investigated in this study. Firstly, both *T. oreinos* subspecies can be assigned unequivocally based on their barcodes (maximum 1.4% distance within subspecies and 13.7% between subspecies). Secondly, nearly the whole distribution range of these two taxa was investigated. Concerning *T. striolatus*, all individuals ($n = 51$) morphologically assigned to this species are monophyletic and display low intraspecific variation (maximum 3.7%) over a quite large distribution range. The nearest relative in our tree (*T. villosus*) is separated by 9.5%.

With respect to *T. biconicus*, *T. villosus*, *T. clandestinus* and *T. villosulus*, the *COI* data of the present investigation are in accordance with those of Pfenninger *et al.* (2005). Each of these species is monophyletic, and in a pooled data set, the maximum intraspecific distances are low: *T. biconicus* (2%), *T. villosus* (2.7%), *T. clandestinus* (0.3%) and *T. villosulus* (2.1%). Therefore, these species should be easily determinable by DNA barcoding.

Conclusions

The most striking outcome of the present study is the very well-supported paraphyly revealed in the mtDNA phylogeny of the *T. hispidus* complex. None of the nine mtDNA clades shows a morphological differentiation; however, it remains questionable if any of them might represent cryptic species. Therefore, gene flow has to be tested in populations where representatives of multiple clades can be found. The presence of morphologically well-defined species within the complex, together with possible cryptic species, suggests that budding speciation in the genus *Trochulus* occurred frequently. The *T. hispidus* complex is a very prominent example of a species in which, despite the intense investigation, species delimitation remains unclear. This makes further analysis even more interesting because in the process of speciation, the mechanisms (such as hybridisation) that provoke such a pattern are still not sufficiently understood.

Acknowledgements

We thank the Austrian Science Foundation for financial support of this work (FWF Project No. 19592-B17). The 'Friends of the Museum of Natural History Vienna' provided financial support for travel expenses. Zoltan Feher, Ted von Proschwitz, Ira Richling and Ulrich Schneppat provided important samples from regions outside the Eastern Alps. For help during collection trips, special thanks goes to Helmut Baminger, Barbara Däubl, Agnes Bisenberger, Philipp Haselwanter, Christoph Hörweg, Paul Mildner, Laura Zopp, Wilhelm Pinsker, Anatoly Schileyko, Reiko Slapnik, Erich Weigand and Sabine Zwierschitz. Furthermore, we thank Laura Zopp for archiving the specimens and maintaining the database. We are very grateful to Wilhelm Pinsker for many valuable discussions and critical comments on the article. The file with the LGM glacier line was generously provided by Peter Schönswetter and was first published in (Schönswetter *et al.* 2002).

References

Avise, J. (2000). *Phylogeography: The History and Formation of Species*. Cambridge, MA and London: Harvard University Press.

Bandelt, H. J., Forster, P. & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, *16*, 37–48.

Binder, H. (1977). Bemerkenswerte Molluskenfaua aus dem Pliozän und Pleistozän von Niederösterreich. *Beiträge zur Paläontologie von Österreich*, *3*, 1–78.

Cadahía, L., Harl, J., Duda, M., Sattmann, H., Kruckenhauser, L., Fehér, Z., Zopp, L. & Haring, E. (2013). New data on the phylogeny of Ariantinae (Pulmonata, Helicidae) and the systematic position of *Cylindrus obtusus* based on nuclear and mitochondrial DNA marker sequences. *Journal of Zoological Systematics and Evolutionary Research*, in press. doi: 10.1111/jzs.12044.

Capella-Gutierrez, S., Silla-Martinez, J. M. & Gabaldon, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics*, *25*, 1972–1973.

Cracraft, J. (1983). Species concepts and speciation analysis. *Current Ornithology*, *1*, 159–187.

Davison, A., Blackie, R. L. E. & Scothern, G. P. (2009). DNA barcoding of stylommatophoran land snails: a test of existing sequences. *Molecular Ecology Resources*, *9*, 1092–1101.

Depraz, A., Hausser, J. & Pfenninger, M. (2009). A species delimitation approach in the *Trochulus sericeus/hispidus* complex reveals two cryptic species within a sharp contact zone. *BMC Evolutionary Biology*, *9*, 171.

Dillon, R. T. & Robinson, J. D. (2008). The snails the dinosaurs saw: are the pleurocerid populations of the Older Appalachians a relict of the Paleozoic Era? *Journal of the North American Benthological Society*, *28*, 1–11.

Duda, M., Kruckenhauser, L., Haring, E. & Sattmann, H. (2010). Habitat requirements of the pulmonate land snails *Trochulus oreinos oreinos* and *Cylindrus obtusus* endemic to the Northern Calcareous Alps, Austria. *eco.mont - Journal on Protected Mountain Areas Research and Management*, *2*, 5–12.

Duda, M., Sattmann, H., Haring, E., Bartel, D., Winkler, H., Harl, J. & Kruckenhauser, L. (2011). Genetic differentiation and shell morphology of *Trochulus oreinos* (Wagner, 1915) and *T. hispidus* (Linnaeus, 1758) (Pulmonata: Hygromiidae) in the Northeastern Alps. *Journal of Molluscan Studies*, 77, 30–40.

Duda, M., Kruckenhauser, L., Sattmann, H., Harl, J. & Haring, E. (revised). Morphological and ecological differentiation in hairy snails (Gastropoda, Pulmonata, Hygromiidae). *Journal of Molluscan Studies*.

Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, *1*, 47–50.

Falkner, G. (1982). Zur Problematik der Gattung *Trichia* (Pulmonata, Helicidae) in Mitteleuropa. *Mitteilungen der Deutschen Malakozoologischen Gesellschaft*, *3*, 30–33.

Forcart, L. (1965). New researches on *Trichia hispida* (Linnaeus) and related forms (pp. 79–93). Presented at the Proceedings of the first European Malacological Congress, London.

Frank, C. (2006). *Plio-pleistozäne und holozäne Mollusken Österreichs, Teil 2*. Vienna: Verlag der Österreichischen Akademie der Wissenschaften.

Frank, C., Terhorst, B., Damm, B., Thiel, C., Frechen, M. & Peticzka, R. (2011). Pleistocene loess deposits and mollusc assemblages in the Eastern Pre-Alps. *Quaternary Science Journal*, *60*, 126–136.

Funk, D. J. & Omland, K. E. (2003). Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology, Evolution, and Systematics*, *34*, 397–423.

Gittenberger, E., Piel, W. H. & Groenenberg, D. S. J. (2004). The Pleistocene glaciations and the evolutionary history of the polytypic snail species *Arianta arbustorum* (Gastropoda, Pulmonata, Helicidae). *Molecular Phylogenetics and Evolution*, *30*, 64–73.

Haase, M., Misof, B., Wirth, T., Baminger, H. & Baur, B. (2003). Mitochondrial differentiation in a polymorphic land snail: evidence for Pleistocene survival within the boundaries of permafrost. *Journal of Evolutionary Biology*, *16*, 415–428.

Haase, M., Esch, S. & Misof, B. (2013). Local adaptation, refugial isolation and secondary contact of Alpine populations of the land snail *Arianta arbustorum*. *Journal of Molluscan Studies*, *79*, 241–248.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hayashi, M. & Chiba, S. (2000). Intraspecific diversity of mitochondrial DNA in the land snail *Euhadra peliomphala* (Bradybaenidae). *Biological Journal of the Linnean Society*, *70*, 391–401.

Huelsenbeck, J. & Ronquist, F. (2001). MrBayes: bayesian inference of phylogeny. *Bioinformatics*, *17*, 754–755.

Kerney, M., Cameron, R. & Jungbluth, J. (1983). *Die Landschnecken Nord- und Mitteleuropas*. Hamburg, Berlin: Paul Parey Verlag.

Kruckenhauser, L., Harl, J. & Sattmann, H. (2011). Optimized drowning procedures of pulmonate land snails allowing subsequent DNA analysis and anatomical dissections. *Annalen des Naturhistorischen Museums in Wien*, *Serie B*, *112B*, 173–175.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, *23*, 2947–2948.

Li, W. H. & Graur, D. (1991). *Fundamentals of Molecular Evolution*. Sunderland: Sinauer Associates.

Ložek, V. (1956). *Klíč československých měkkýšů*. Bratislava: Vydavatel'stvo slovenskej akadémie vied.

Ložek, V. (1964). Quartärmollusken der Tschechoslowakei. *Rozpravy Ústředního ústavu geologického*, *31*, 1–374.

Marković, S. B., McCoy, W. B., Oches, E. A., Savić, S., Gaudenyi, T., Jovanović, M., Stevens, T., Walther, R., Ivanišević, P. & Galović, Z. (2005). Paleoclimate record in the Upper Pleistocene loess-paleosol sequence at Petrovaradin brickyard (Vojvodina, Serbia). *Geologica Carpathica*, *56*, 545–552.

Mayr, E. (1942). *Systematics and the Origin of Species*. New York: Columbia University Press.

Mayr, E. (1970). *Populations, Species, and Evolution*. Cambridge: Harvard University Press.

Mikula, E. (1957). *Trochulus hispidus* scheerpeltzi *n. subsp*. *Archiv für Molluskenkunde*, *86*, 91–93.

Molnár, D., Hupuczi, J., Galović, L. & Sümegi, P. (2010). Preliminary malacological investigation on the loess profile at Zmajevac, Croatia. *Central European Journal of Geosciences*, *2*, 52–56.

Murray, J. (1964). Multiple Mating and Effective Population Size in *Cepaea nemoralis*. *Evolution*, *18*, 283–291.

Nixon, K. C. & Wheeler, Q. D. (1992). Extinction and the Origin of Species. In M. J. Novacek & Q. D. Wheeler (Eds) *Extinction and Phylogeny* (pp. 119–143). New York: Columbia University Press.

Pfenninger, M. & Pfenninger, A. (2005). A new *Trochulus* species from Switzerland (Gastropoda: Pulmonata: Hygromiidae). *Archiv für Molluskenkunde*, *134*, 261–269.

Pfenninger, M., Posada, D. & Magnin, F. (2003). Evidence for survival of Pleistocene climatic changes in Northern refugia by the land snail *Trochoidea geyeri* (Soós 1926) (Helicellinae, Stylommatophora). *BMC Evolutionary Biology*, *3*, 8.

Pfenninger, M., Hrabáková, M., Steinke, D. & Dèpraz, A. (2005). Why do snails have hairs? A Bayesian inference of character evolution *BMC Evolutionary Biology*, *5*, 59.

Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, *25*, 1253–1256.

Prévot, V., Jordaens, K., Sonet, G. & Backeljau, T. (2013). Exploring species level taxonomy and species delimitation methods in the facultatively self-fertilizing land snail genus *Rumina* (Gastropoda: Pulmonata) *PLoS ONE*, *8*, e60736.

Proćków, M. (2009). The genus *Trochulus* Chemnitz, 1786 (Gastropoda: Pulmonata: Hygromiidae) - a taxonomic revision. *Folia Malacologica*, *17*, 101–176.

Proćków, M., Drvotová, M., Juřičková, L. & Kuźnik-Kowalska, E. (2013). Field and laboratory studies on the life-cycle, growth and feeding preference in the hairy snail *Trochulus hispidus* (L., 1758) (Gastropoda: Pulmonata: Hygromiidae). *Biologia*, *68*, 131–141.

Rabitsch, W., Essl, F., Allspach, A. & Frajman, B. (2009). *Endemiten: Kostbarkeiten in Österreichs Pflanzen- und Tierwelt*. Naturwiss: Verein für Kärnten.

Rawson, P. D. & Burton, R. S. (2002). Functional coadaptation between cytochrome c and cytochrome c oxidase within allopatric populations of a marine copepod. *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 12955–12958.

Rousseau, D. D., Puissegur, J. J. & Lecolle, F. (1992). West-European terrestrial mollusks assemblages of isotopic stage-11 (Middle Pleistocene) - climatic implications. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *92*, 15–29.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406–425.

Sánchez, R., Serra, F., Tárraga, J., Medina, I., Carbonell, J., Pulido, L., de María, A., Capella-Gutíerrez, S., Huerta-Cepas, J., Gabaldón, T., Dopazo, J. & Dopazo, H. (2011). Phylemon 2.0: a suite of web-tools for molecular evolution, phylogenetics, phylogenomics and hypotheses testing. *Nucleic Acids Research*, *39*, (Web Server issue), W470–W474.

Sauer, J. & Hausdorf, B. (2009). Sexual selection is involved in speciation in a land snail radiation on crete. *Evolution*, *63*, 2535–2546.

Sauer, J. & Hausdorf, B. (2010). Reconstructing the evolutionary history of the radiation of the land snail genus *Xerocrassa* on Crete based on mitochondrial sequences and AFLP markers. *BMC Evolutionary Biology*, *10*, 299.

Sauer, J. & Hausdorf, B. (2012). A comparison of DNA-based methods for delimiting species in a Cretan land snail radiation reveals shortcomings of exclusively molecular taxonomy. *Cladistics*, *28*, 300–316.

Scheel, B. M. & Hausdorf, B. (2012). Survival and differentiation of subspecies of the land snail *Charpentieria itala* in mountain refuges in the Southern Alps. *Molecular Ecology*, *21*, 3794–3808.

Schileyko, A. (1978). On the systematics of *Trichia* s. lat. (Pulmonata: Helicoidea: Hygromiidae). *Malacologia*, *17*, 1–56.

Schönswetter, P., Tribsch, A., Barfuss, M. & Niklfeld, H. (2002). Several Pleistocene refugia detected in the high alpine plant *Phyteuma globulariifolium* Sternb. & Hoppe (Campanulaceae) in the European Alps. *Molecular Ecology*, *11*, 2637–2647.

Schönswetter, P., Stehlik, I., Holderegger, R. & Tribsch, A. (2005). Molecular evidence for glacial refugia of mountain plants in the European Alps. *Molecular Ecology*, *14*, 3547–3555.

Sümegi, P., Gulyás, S., Persaits, G., Gergely Páll, D. & Molnár, D. (2011). The loess-paleosol sequence of Basaharc (Hungary) revisited: mollusc-based paleoecological results for the Middle and Upper Pleistocene. *Quaternary International*, *240*, 181–192.

Sysoev, A. V. & Schileyko, A. A. (2009). *Land Snails of Russia and Adjacent Countries*. Sofia & Moscow: Pensoft.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*, 1596–1599.

Thomaz, D., Guiller, A. & Clarke, B. (1996). Extreme divergence of mitochondrial DNA within species of pulmonate land snails. *Proceedings of the Royal Society B: Biological Sciences*, *263*, 363–368.

Toussaint, E. F. A., Sagata, K., Surbakti, S., Hendrich, L. & Balke, M. (2013). Australasian sky islands act as a diversity pump facilitating peripheral speciation and complex reversal from narrow endemic to widespread ecological supertramp. *Ecology and Evolution*, *3*, 1031–1049.

Tribsch, A. & Schönswetter, P. (2003). Patterns of endemism and comparative phylogeography confirm palaeoenvironmental evidence for Pleistocene refugia in the Eastern Alps. *Taxon*, *52*, 477–497.

Van Husen, D. (1997). LGM and late-glacial fluctuations in the Eastern Alps. *Quaternary International*, *38–39*, 109–118.

Van Riel, P., Jordaens, K., Van Houtte, N., Frias Martins, A. M., Verhagen, R. & Backeljau, T. (2005). Molecular systematics of the endemic Leptaxini (Gastropoda: Pulmonata) on the Azores islands. *Molecular Phylogenetics and Evolution*, *37*, 132–143.

Vanderpoorten, A. & Long, D. G. (2006). Budding speciation and neotropical origin of the Azorean endemic liverwort, *Leptoscyphus azoricus*. *Molecular Phylogenetics and Evolution*, *40*, 73–83.

Wade, C. M. & Mordan, P. B. (2000). Evolution within the gastropod molluscs; using the ribosomal RNA gene-cluster as an indicator of phylogenetic relationships. *Journal of Molluscan Studies*, *66*, 565–570.

Wagner, A. J. (1915). Beiträge zur Anatomie und Systematik der Stylomatophoren aus dem Gebiet der Monarchie und der angrenzenden Balkanländer. *Denkschriften der Österreichischen Akademie der Wissenschaften (mathematisch-naturwissenschaftliche Klasse)*, *91*, 429–498.

Welter-Schultes, F. (2012). *European Non-Marine Molluscs, a Guide for Species Identification*. Göttingen: Planet Poster Editions.

Willett, C. S. (2006). Deleterious epistatic interactions between electron transport system protein-coding loci in the copepod *Tigriopus californicus*. *Genetics*, *173*, 1465–1477.

Xia, X. & Xie, Z. (2001). DAMBE: Data Analysis in Molecular Biology and Evolution (version 4.2.0.13). *Journal of Heredity*, *92*, 371–373.

Xia, X., Xie, Z., Salemi, M., Chen, L. & Wang, Y. (2003). An index of substitution saturation and its application. *Molecular Phylogenetics and Evolution*, *26*, 1–7.

Zachos, F. E. (2009). Gene trees and species trees – mutual influences and interdependences of population genetics and systematics. *Journal of Zoological Systematics and Evolutionary Research*, *47*, 209–218.

Zachos, F. E., Apollonio, M., Bärmann, E. V., Festa-Bianchet, M., Göhlich, U., Habel, J. C., Haring, E., Kruckenhauser, L., Lovari, S., McDevitt, A. D., Pertoldi, C., Rössner, G. E., Sánchez-Villagra, M. R., Scandura, M. & Suchentrunk, F. (2013). Species inflation and taxonomic artefacts—A critical

comment on recent trends in mammalian classification. *Mammalian Biology*, *78*, 1–6.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Neighbour-joining tree of the *COI* sequences from all individuals providing an overview of the different clades, subclades and species. The height of the triangles represents the number of individuals found in each clade, and the depth of the triangle reflects the divergence of haplotypes in each clade. Black dots indicate maximum support. Bootstrap support above 75% is indicated. Note that the tree contains only little information about the relationships between the clades. The colour code is the same as in Fig. 1. Details are given in Figs S2–S4.

Fig. S2. Partial tree of the neighbour-joining tree of the *COI* sequences (Fig. S1) illustrating the clade 2 in detail. Each individual is defined by an individual Id, the origin of geographic region and the Id of the sampling sites. For relevant nodes, bootstrap values are indicated.

Fig. S3. Partial tree of the neighbour-joining tree of the *COI* sequences (Fig. S1) illustrating the various clades and subclades in detail: Clades 3 to 7 as well as *T. coelomphala*, *T. striolatus*, and *T. villosulus*. Each individual is defined by an individual Id, the origin of geographic region and the Id of the sampling sites. For relevant nodes, bootstrap values are indicated. The haplogroups (hg1–hg5) and subspecies of *T. striolatus* are indicated.

Fig. S4. Partial tree of the neighbour-joining tree of the *COI* sequences (Fig. S1) illustrating the various clades and subclades in detail: Clades 1, 8, and 9 as well as the species *T. clandestinus*, *T. oreinos*, *T. biconicus*, *T. villosus*, *Monacha cantiana*, *Plicuteria lubomirski*, and *Isognomostoma isognomostomos*. Each individual is defined by an individual Id, the origin of geographic region and the Id of the sampling sites. For relevant nodes, bootstrap values are indicated.

Table S1. Summarising information for the individuals investigated in this study together with GenBank accession numbers and coordinates of sampling sites. Individual Id (indId), Geographic origin (region), sampling site ID (spID), Country (cn). Abbreviations of clades as in Fig. 1 and Is_iso (*Isognomostoma isognomostomos*); Mo_can (*Monacha cantiana*); Pl_lub (*Plicuteria lubomirski*); Clade numbers correspond to those in Figs. 1, 2 and 4.

Table S2. Mean and maximum genetic p-distances (in %) between *COI* sequences within species and clades of *T. hispidus* (upper two lines) and mean p-distances between species and clades. Abbreviations of clades as in Fig. 1 and Pl_lub (*Plicuteria lubomirski*); Mo_can (*Monacha cantiana*); Is_iso (*Isognomostoma isognomostomos*). Clade numbers correspond to those in Figs. 1, 2 and 4.

