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DISSERTATION

Titel der Dissertation

Phylogeography and taxonomy of the land snail genus *Orcula* Held, 1837

Verfasser

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Preface

This doctoral thesis is a cumulative dissertation, compiling three publications, all of which were published in peer-reviewed journals. The publications deal with taxonomy, phylogeny and phylogeography of the land snail genus *Orcula* Held, 1837. The general introduction of the thesis provides a background on the suitability of land snails for evolutionary questions, in particular regarding the search for glacial refuges. The next section contains my three first-author publications, which are co-authored (in varying combinations) by members of the research group on Alpine land snails, which is housed in the Natural History Museum Vienna, Elisabeth Haring, Helmut Sattmann, Luise Kruckenhauser, Michael Duda, Sandra Kirchner, and two malacologists from other institutions, Barna Páll-Gergely and Anatoly Schileyko. Elisabeth Haring, Helmut Sattmann and Luise Kruckenhauser initiated the project on the diversity of Alpine land snails and participated in the design of my studies. The latter three researchers and my colleague, Michael Duda, were equally involved in fieldwork and sample collection. For all three studies, I performed the main part of the lab work, data analyses, interpretation and writing. Anatoly Schileyko conceived the idea of the type-catalogue and essentially contributed in writing the manuscript. Barna Páll-Gergely's efforts and expertise were crucial for completing the taxon set for the phylogeny of the genus *Orcula*. Detailed information on contributions of the authors are provided in the 'Authors' Contributions'. The main part of my thesis is followed by a concluding discussion containing chapters on glacial refuges, systematics and hybridization as well as a section on methodological considerations, mainly about the choice of molecular genetic markers and primer design.

Summary

This doctoral thesis deals with taxonomy, phylogeny and phylogeography of the land snail genus *Orcula* Held, 1837. The genus *Orcula* inhabits calcareous mountain habitats of Central and Southeast Europe and shows the largest species diversity in the Eastern Alps with nine out of 13 species. Several malacologists hypothesized that the high diversity in the Alps was the result of geographic isolation of populations in separated glacial refuges and adaption to changing environmental conditions and that particularly the Eastern Alps represented a center of origin and glacial refuge for several taxa. For the present thesis, we studied the taxonomy of the genus and investigated all *Orcula* species by means of molecular genetics. We integrated fossil data for reconstructing past distribution patterns and for calibrating the phylogenetic trees, and we performed morphometric analyses on members of the Alpine species group. In particular, we aimed at clarifying whether the high species and subspecies diversity in the Eastern Alps is the result of geographical isolation in Pleistocene glacial refuges or if if the lineages already separated in earlier times.

In the type catalogue, we provide comprehensive data on all extant taxa of the genus *Orcula*. We identified 55 nominal taxa*,* for most of which we provide references to the original descriptions, type localities, places of storage of type specimens and collection numbers. For more than 30 nominal taxa we also present photographs and measurements of type specimens. We discuss the validity of several subspecies names and point out inconsistencies in taxon listings of the Fauna Europaea Checklist and the CLECOM I-list. This work allowed us to identify all relevant taxa and to evaluate their taxonomical *status quo*, which was crucial for any further investigations.

The phylogenetic trees resulting from the analyses of both the mitochondrial (*COI*, *12S*, *16S*) and the nuclear (*H4*/*H3*) data sets reveal three main clades, corresponding to the three subgenera *Orcula*, *Illyriobanatica* Páll-Gergely & Deli 2013 and *Hausdorfia* Páll-Gergely & Irikov 2013. The fossil calibrated molecular clock analyses and the reconstructions of the historic geographic ranges suggest that the genus originated in the Dinarids during the Middle Miocene and that the subgenus *Orcula* colonized the Alps not until the Late Miocene. The major splits in the latter species group date back to the Late Miocene and Pliocene, therefore, isolation in separated Pleistocene glacial refuges was most likely not the only factor triggering speciation. Our data also suggest that hybridizations happened or are still ongoing between some of the 'younger' species, particularly between *Orcula pseudodolium* Wagner 1912 and *Orcula gularis* (Rossmässler, 1837). The two latter species could be discriminated well in the morphometric analyses and generally provided different nuclear *H4*/*H3* variants, but almost all specimens possessed similar mitochondrial sequences, thus indicating mitochondrial capture.

Complex phylogeographic patterns were also found in the most widespread and prominent *Orcula* species, *Orcula dolium* (Draparnaud, 1801). The species inhabits all major limestone areas of the Alps and the Western Carpathians and includes several morphologically differentiated populations. The phylogenetic trees reveal at least four distinct Alpine clades, whose

distribution areas are overlapping only marginally. These clades, however, could not be related to any of the nominate subspecies, except for a single clade, which matches with the distribution of *Orcula dolium infima* Ehrmann, 1933 from the Wienerwald (Vienna and Lower Austria). *Orcula dolium edita* Ehrmann, 1933 and *Orcula dolium raxae* Gittenberger, 1978, both described as glacial relics, which are currently restricted to high altitudes in the Eastern Alps, were genetically not distinguishable from the surrounding lowland populations. Nonetheless, the phylogeographic patterns indicate that populations of *O. dolium* outlasted the Last Glacial Maximum in refuges in the Western Alps and in the Eastern Alps - in particular, the latter region harbors genetically highly diverse populations. Despite the considerably smaller sample size, we found an even larger number of genetic clades in the Western Carpathians, and specimens at almost half of the sites showed strongly differing mitochondrial sequences. The reconstruction of the geographic range history suggests that *O. dolium* originated in the Western Carpathians around the Miocene-Pliocene boundary and settled the Alps later. The Alpine populations, however, are not reciprocally monophyletic, but sequence patterns suggest multiple migrations between the two mountain ranges. In order to reconstruct past distribution patterns of *O. dolium* during the last glacial periods in lowland areas surrounding the Alps, we also examined the Pleistocene fossil record of the species. The data clearly evidences its presence in the periphery of the Alps and the Western Carpathians, as well as in the Pannonian and Vienna Basins, during several warm and cold glacial periods.

Zusammenfassung

Die vorliegende Doktorarbeit behandelt die Taxonomie, Phylogenie und Phylogeographie der Landschneckengattung *Orcula* Held, 1837. Die Gattung *Orcula* bewohnt kalkreiche Gebirgshabitate Zentral- und Südosteuropas und weist mit neun von 13 Arten die größte Artenvielfalt in den Ostalpen auf. Die hohe Vielfalt in den Alpen wurde von mehreren Autoren auf die geographische Isolation von Populationen in voneinander getrennten eiszeitliche Refugien und auf die Anpassung an sich ändernde Umweltbedingungen zurückgeführt - insbesondere die Ostalpen wurden als Entstehungszentrum und eiszeitliches Refugium vieler Taxa vermutet. Im Rahmen dieser Dissertation führten wir Studien zur Taxonomie der Gattung sowie molekulargenetische Untersuchungen bei allen *Orcula*-Arten durch. Für die Rekonstruktion vergangener Verbreitungsmuster und die Kalibrierung der phylogenetischen Stammbäume wurden Fossilienbelege mit einbezogen, und bei den Mitgliedern der alpinen Artengruppe wurden morphometrische Analysen durchgeführt. Insbesondere strebten wir an zu klären, ob die hohe Arten- und Unterartenvielfalt in den östlichen Alpen das Resultat von Isolation in eiszeitlichen Refugien während des Pleistozäns ist oder ob sich deren Abstammungslinien bereits davor trennten.

Im Typenkatalog stellen wir umfangreiche Daten zu allen bestehenden Arten der Gattung *Orcula* zur Verfügung. Wir identifizierten 55 nominelle Taxa, für einen Großteil derer wir Referenzen zu den Originalbeschreibungen, Typus-Lokalitäten, Aufbewahrungsorten von Typus-Exemplaren und Sammlungsnummern auflisten. Des Weiteren zeigen wir Abbildungen und Abmessungen von mehr als 30 nominellen Taxa, diskutieren die Gültigkeit mehrerer Unterartnamen und weisen auf Inkonsistenzen in den Auflistungen der Taxa in der Fauna-Europaea und der CLECOM I-Liste hin. Diese Arbeit ermöglichte uns, alle relevanten Taxa zu identifizieren, was entscheidend für die weiteren Untersuchungen war.

Die Stammbäume, die aus der Untersuchung sowohl der mitochondrialen (*COI*, *12S*, *16S*) als auch der nukleären (*H4/H3*) Datensätze resultierten, zeigen drei Kladen, die den drei Untergattungen *Orcula*, *Illyriobanatica* Páll-Gergely & Deli 2013 und *Hausdorfia* Páll-Gergely & Irikov 2013 entsprechen. Die durch Fossilien kalibrierten 'Molekulare Uhr'-Analysen und die Rekonstruktion der historischen Verbreitungen deuten darauf hin, daß die Gattung bereits während des Mittleren Miozäns im Dinarischen Gebirge entstanden ist und die Untergattung *Orcula* die Alpen nicht vor dem späten Miozän besiedelt hat. Die Auftrennung der wichtigsten Linien innerhalb dieser Artengruppe datieren auf das Obere Miozän und das Pliozän - die Isolation in getrennten eiszeitliche Refugien war wahrscheinlich nicht der einzige Faktor, der die Artbildung vorantrieb. Unsere Daten weisen auch darauf hin, daß es zu Hybridisierungen gekommen ist oder diese zwischen einigen 'jüngeren' Arten immer noch auftreten, insbesondere zwischen *Orcula pseudodolium* Wagner, 1912 und *Orcula gularis* (Rossmässler, 1837). Die beiden letzteren Arten konnten in den morphometrischen Analysen gut unterschieden werden und wiesen generell unterschiedliche nukleäre *H4/H3*-Varianten auf, aber fast alle Individuen besaßen

ähnliche mitochondriale Sequenzen, was auf die Aufnahme artfremder mitochondrialer DNA ('mitochondrial capture') hindeutet.

Komplexe phylogeographische Muster wurden auch bei der am weitesten verbreiteten und auffälligsten *Orcula*-Art, *Orcula dolium* (Draparnaud, 1801), gefunden. Die Art besiedelt alle größeren Kalksteingebiete der Alpen und der westlichen Karpaten und umfaßt mehrere morphologisch differenzierte Populationen. Die phylogenetischen Bäume zeigen zumindest vier unterschiedliche alpine Kladen, deren Verbreitungsgebiete nur geringfügig überlappen. Diese Kladen konnten allerdings nicht mit den nominellen Unterarten in Verbindung gebracht werden, außer einer einzigen Klade, die mit dem Verbreitungsareal von *Orcula dolium infima* Ehrmann, 1933 aus dem Wienerwald (Wien und Niederösterreich) übereinstimmt. *Orcula dolium edita* Ehrmann, 1933 und *Orcula dolium raxae* Gittenberger, 1978, die als eiszeitliche Relikte beschrieben wurden, die heute auf höhere Gebirgslagen der östlichen Alpen beschränkt sind, konnten genetisch nicht von den umgebenden Populationen des Tieflandes unterschieden werden. Die phylogeographischen Muster deuten dennoch darauf hin, daß *O. dolium* das letzte glaziale Maximum in Refugien in den West- und den Ostalpen überdauerte - insbesondere die Ostalpen beherbergen genetisch sehr diverse Populationen. Ungeachtet der wesentlich kleineren Probenanzahl wurde in den Westkarpaten sogar eine noch größere Zahl an genetischen Kladen gefunden, und die Individuen von annähernd der Hälfte der Standorte wiesen stark unterschiedliche mitochondriale Sequenzen auf. Die Rekonstruktion der geographischen Verbreitungsgeschichte deutet darauf hin, daß *O. dolium* etwa um die Miozän-Pliozän-Grenze in den Westkarpaten entstand und die Alpen erst später besiedelte. Die alpinen Populationen sind allerdings nicht reziprok monophyletisch - die Sequenzmuster weisen auf mehrfache Migrationen zwischen den beiden Gebirgszügen hin. Um die vergangenen Verbreitungsmuster von *O. dolium* während der letzten glazialen Abschnitte zu rekonstruieren, untersuchten wir auch die pleistozänen Fossilienbelege der Art. Die Daten zeigen klar die Anwesenheit von *O. dolium* in der Peripherie der Alpen und Westkarpaten sowie im Pannonischen und im Wiener Becken während mehrerer kalter und warmer eiszeitlicher Perioden.

General Introduction

Already more than half a century ago Holdhaus (1954) proposed that several Alpine species survived cold Pleistocene periods in glacial refuges in the Alps, and the topic is still a matter of debate in an increasing number of publications. Holdhaus (1954) assumed that glacial refuges of blind troglobiotic beetles and other endemic animal and plant species were located at the periphery of the Western Alps and the Southern Calcareous Alps. Further refuges of troglomorphic ground beetle species were proposed later for the eastern part of the Northern Calcareous Alps by Daffner (1993) and Homburg (2013). The Northern and the Southern Calcareous Alps also harbor a high number of endemic land snails (Klemm, 1974). Moreover, Late Pleistocene loess sediments provide good evidence that various Alpine gastropod species outlasted the Last Glacial Maximum at the periphery of the Western and Eastern Alps (Moine et al., 2005; Frank et al., 2011), in the Western Carpathians (Ložek, 1964) and in the Pannonian Basin (Főköh et al., 1995). Among gastropods frequently found in Late Pleistocene loess sediments are extant species, which are common in the Alps, e.g., *Trochulus hispidus* (Linné, 1758), *Clausilia dubia* Draparnaud, 1805, *Arianta arbustorum* (Linnaeus, 1758) and *O. dolium*. During cold glacial periods, these species probably inhabited un-glaciated regions of the Alps as well, but the local fossil record is scarce since conditions for fossilization are unfavorable in mountain areas. Therefore, the identification of glacial refuges located in Alpine mountain regions is complicated and usually not possible with fossil data alone.

Advances in molecular genetics during the last decades, in particular the introduction of the polymerase chain reaction (PCR) by Mullis et al. (1986), opened up entirely new opportunities for studying phylogeography of organisms. A considerably high number of papers aimed at evaluating the influence of the Pleistocene glaciations on distribution and genetic diversity of European biota. A central theme in most of these studies is the search for glacial refuges. Generally, populations of former refuge areas are assumed to have diverged during isolation in geographically separated areas and, therefore, are characterized by the presence of rare (private) alleles and high genetic diversity (Provan & Bennett, 2008). Around the turn of the millennium Taberlet et al. (1998) and Hewitt (1999, 2000, 2004) published first review articles about the genetic consequences of the quarternary climate oscillations on European biota. The revision of molecular genetic data of several vertebrates, plants and a grasshopper species let them conclude that the respective refuge areas were mainly located in Southern Europe. The Alps, however, were not considered as potential refuge area for the latter taxa. Later, molecular genetic analyses were extensively performed on a wide range of other taxa, leading to a fundamentally new understanding of the biogeography of Alpine species. With more than a dozen species investigated, potential refuges of Alpine plants restricted to high mountain areas are well characterized. These studies provide strong support for the existence of glacial refuges in the Eastern and in the Western Alps (summarized in Tribsch & Schönswetter, 2003; Schönswetter et al., 2005). A few studies also aimed at identifying glacial refuges of terrestrial land snails. Glacial refuges of *Trochulus villosus* (Draparnaud, 1805) were probably located in the Western Alps

(Dépraz et al., 2008), and those of two *Carychium* species, *C. minimum* O.F. Müller, 1774 and *C. tridentatum* (Risso, 1826), in both the Western and the Eastern Alps (Weigand et al., 2012). Eastern Alpine refuges were also assumed for *Arianta arbustorum* (Linnaeus, 1758) (Gittenberger et al., 2004; Haase et al., 2013). High endemism rates at the margins of the Western and Eastern Alps further indicate that these areas represented glacial refuges (Tribsch & Schönswetter, 2003; Rabitsch et al., 2009).

In respect to the search of glacial refuges, phylogeographic studies of land snails have the potential to contribute much to our knowledge. Many species show specific habitat preferences and low active dispersal capacities, which generally makes them suitable model organisms for studies of speciation, habitat fragmentation and radiation (Glaubrecht, 2009). High population densities enable the collection of adequate specimen numbers in many species, which facilitates population genetics and morphological studies. Moreover, gastropod shells show high abundances in the fossil record, which allows deducing past distribution patterns and tracing changes in shell morphology over time.

For this doctoral thesis, we studied the phylogeography of all members of the land snail genus *Orcula*, with a special emphasis on the search for glacial refuges of the Alpine species. The genus *Orcula* belongs to the family Orculidae (Pulmonata, Stylommatophora) and currently includes 13 species, which belong to three subgenera. The subgenus *Illyriobanatica* includes only two or three species, which inhabit the Dinarids and the Southern Carpathians, respectively, whereas the subgenus *Hausdorfia* is monotypic and distributed in the western Black Sea region. The subgenus *Orcula* comprises nine species, seven of which are strictly calciphilous and endemic to limestone areas of the Eastern Alps. *O. conica* (Rossmässler, 1837) is mainly distributed in the Eastern Alps, but was found also at the Croatian Plitvice lakes. Apart from the latter eight species, the subgenus *Orcula* includes the genus' most widespread species, *O. dolium*, whose distribution extends from the Western Alps to the Western Carpathians and covers an altitudinal range from 200 m to 2200 m above sea level. Several authors previously studying the Alpine species emphasized that the formation of species and subspecies populations in the Eastern Alps was most likely linked with adaption to cold climates and isolation in separated glacial refuges during the Pleistocene (Zimmermann, 1932; Klemm, 1967, 1974; Frank, 2006). In the present thesis, we aimed at identifying glacial refuges of the Alpine *Orcula* species by means of molecular genetics and, in *O. dolium*, also by examining the fossil record. Moreover, we wanted to elicit whether the formation of the *Orcula* species and subspecies was only the result of the Pleistocene climate changes or dates back to earlier geological epochs.

Publications

Harl J, Sattmann H, Schileyko, A (2011) Types of the extant taxa of the landsnail genus *Orcula* Held 1837 (Gastropoda: Stylommatophora: Orculidae). *Archiv für Molluskenkunde - International Journal of Malacology*, **140**, 175-199.

Types of the extant taxa of the landsnail genus *Orcula* Held 1837

(Gastropoda: Stylommatophora: Orculidae)

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Abstract

The aim of this paper is to reflect the status quo of the taxonomical situation of *Orcula* Held 1837 which we assume to be a prerequisite for a thorough revision of the whole genus. Illustrated data on type specimens of more than 30 nominal taxa of the genus *Orcula* are presented. For most taxa the reference to the original description, type locality, place of storage, collection number, nomenclatural status of specimens, measurements of type specimens, and photos of the shells are given. The nomenclatural status and the availability of the taxon names are discussed as well as other important information on the taxa are presented. The selection of taxon subsets for international taxon lists such as the Fauna Europaea Checklist is discussed and different perspectives are shown.

Key words: *Orcula*, Orculidae, Gastropoda, types, descriptions, pictures, nomenclature.

Introduction

At present, the taxonomy of the genus *Orcula* Held 1837, is confusing and inconsistent. At least 50 names exist for the current taxa. A minimum of 14 species is known, four of them described from Austrian territory, three from Slovenia, two from Romania and one each from Albania, Bulgaria, France, Italy, and Montenegro. The phylogenetic position of *Orcula* within the Orculidae is not yet resolved; so far two different views were presented by Gittenberger (1982) and Hausdorf (1996), respectively. Studies of great merit concerning the intrageneric systematics of *Orcula* were performed especially by Zimmermann, Klemm, Gittenberger, and Hausdorf. Pilsbry (1922, 1934) first published compilations of all taxa known at that time including English translations of descriptions and relevant taxonomic discussions.

Zimmermann (1932) attempted a revision of the representatives of the genus in Austria and the neighbouring countries. He was the first who presented a paper that included series of specimens from a wider geographical distribution with measurements and more detailed descriptions. In that paper the evolutionary history of the taxa is discussed for the first time. However, Zimmermann considered conchological characters only. Klemm (1967, 1974) made a big effort in collecting specimens and data on distribution that are still the basis for any investigations of the Eastern Alpine members of the genus. Unfortunately, the distribution areas indicated for the infraspecific taxa appear doubtful. Later Gittenberger (1978) analysed the genital anatomy of most alpine species and of some subspecies and evaluated also morphological characters. The results led to some important changes in the nomenclature such as the splitting of taxa which previously were unified due to conchological similarities. Nevertheless, the status of several

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taxa remained unclear, because sometimes the anatomy led to ambiguous results (e.g., for *O. pseudodolium* or *O. tolminensis*). Moreover, in some cases the analyses were conducted with material that did not stem from the type localities. GITTENBERGER (1978, 1982) also made important comments on the phylogenetic relationships between certain taxa, but the anatomical studies could not resolve the phylogeny of the genus completely. *Orcula* species from the Dinarids, a group previously more or less neglected, were investigated anatomically for the first time by HAUSDORF (1987).

The taxonomy of the infraspecific taxa of the genus *Orcula* is problematic in most cases: The descriptions are sparse and the conchological characters (e.g., shell size and formation of the columellar folds) often vary only slightly between the taxa, but the variability of the shells is very high even within populations. Many descriptions of *O. dolium* subspecies lack clear discrimination criteria and detailed information about the type locality. Moreover, few type localities represent geographically defined areas where the specimens show a constant shell type. Therefore, a practical differentiation of the subspecies, especially of *O. dolium*, is feasible only in a minority of cases.

In the last decade some official listings were published that include members of the genus *Orcula*, such as the Fauna Europaea Checklist (BANK 2004) and the CLECOM I-list (FALKNER et al. 2001). Several incongruities in these lists exemplify the still unsettled taxonomy and systematics of *Orcula*. The authors made a few taxonomical changes and included some taxa and others not. Unfortunately, the reasons that led to these decisions have not been published yet. Concerning some of the subspecies listed in the CLECOM I-list, FALKNER et al. (2001) refer to PILSBRY (1934: 123–133) as author. These taxa (*O. dolium infima*, *O. dolium edita*, and *O. gularis oreina*) originally had been described as "morphae" by ZIMMERMANN (1932: 10). The classification of these taxa is inaccurate as EHRMANN (1933: 50–51) and not PILSBRY (1934) made the names available. A further problem is that ZIMMERMANN's (1932: 10) explanations imply that the "morphae" he described do not represent units of closest relatives or geographical subspecies but that these peculiar shell forms are traits that originated several times independently within the local populations. This raises doubts concerning the subspecific status of these morphotypes. Whether these taxa should be treated as "good" subspecies and consequently be included in official taxon listings remains to be decided after comprehensive morphological and molecular systematic analyses are performed (HARL et al. in prep). However, a problem is that the contents of the taxonomic lists appear arbitrary to some extent as e.g., the French and Slovakian *O. dolium*-subspecies (apart from *O. dolium brancsikii* CLESSIN 1887) are not listed.

In summary, there are many open questions that still remain to be resolved. The aim of this paper is to present a compilation of data on the types of all extant taxa of the genus *Orcula*, with the reference to the original description, type locality, place of storage, collection number, nomenclatural status of specimens, measurements of selected type specimens, and photos of the shells. We located type series of more than 30 taxa in the collections of various museums and, if available, we present pictures of one specimen of each taxon (holotype, syntype, or paratype). For most taxa the nomenclatural status and the availability of the taxon names are discussed and any important information available is presented.

The original descriptions and their English translations will be available on request (see Material and Methods).

This compilation of data on all currently known extant taxa of the genus *Orcula* could help to update commonly used taxon checklists. Most importantly, it is thought to be the first step in an attempt to support future investigations on taxonomy and phylogeny and eventually to facilitate a revision of the whole genus *Orcula*.

Material and methods

We used material of the malacological collections of the "Naturhistorisches Museum, Wien" (NHMW), the "Biologiezentrum der oberösterreichischen Landesmuseen, Linz" (LMOB), the "Naturhistoriska Museum, Göteborg" (MNHG), as well as from the "Naturmuseum Senckenberg, Frankfurt am Main" (SMF). Dr. DOMINIKA MIERZWA from the Museum and Institute of Zoology in Warsaw was contacted twice as parts of the WAGNER collection should be housed in Warsaw but she could not locate any types of the genus *Orcula* in the collection. Requests for collection data to the Muséum national d'histoire naturelle in Paris, the Instytut Biologii Środowiska in Poznan, and the Museo civico di storia naturale in Milano remained unanswered.

In the synonymy list, the assignment of the initial status of each taxon is given according to the original publication ("species", "subspecies", "forma", "morpha", and "variatio"). The taxa are listed in alphabetical order, only the nominative subspecies precede the later described infraspecific taxa. The citation of the original description is given, directly followed by the author. In case the genus name of the taxon was transformed into a new combination, the responsible author and the reference to the literature are placed after the new combination of the taxon, separated by ",–". The synonymy list is followed by the information about the type locality, taken from the type specimens' label or the original description (if no type specimens were available). Since the data sometimes is not complete and names of countries and places changed over time, we tried to provide the contemporary name of the locality and give this additional information in squared brackets. Geographical names in the accessory information are given in original country-specific terms to avoid misinterpretations; additionally, interna-

tional country codes are given (see list of abbreviations). Subsequently, data about the collection numbers of all available material is provided. If specimens are pictured, measurements taken from the photographs are included. Photographs of several focal planes were made with a "Wild Makroskop M420" and a "Nikon DS Camera Control Unit DS-L2". The different layers were combined with "Helicon Focus 4.75 Pro" to obtain one completely focused image. The pictures were cut and edited in "Photoshop CS4". Each specimen was pictured in total vertical aperture view. Moreover, the aperture is pictured in diagonal view, which admits a detailed sight on the aperture's armouring, an important determination criterion within the genus. In addition, for size measurements, the specimens were imaged together with a straight calibration line. Measurements are given from shell height (SH), shell width (SW), aperture height (AH) and aperture width (AW). The numbers of the whorls were counted from pictures in apical and slantwise position. Original descriptions and their English translations are accessible at the homepage of the NHMW (1st Zoological Department; http://snails.nhm-wien.ac.at/publications-congresses <http://webmail/exchweb/bin/redir.asp?URL=http://snails.nhm-wien.ac.at/publications-congresses/>) or can be obtained on request from the corresponding author.

Abbreviations
Shell measurements:

AH   aperture height
AW   aperture width
SH   shell height
SW   shell width

Museums:

LMOB   Biologiezentrum der oberösterreichischen Landesmuseen, Linz
MNHG   Naturhistoriska Museum, Göteborg
MTC   Muzeul "Ţării Crişurilor", Oradea
NHMW   Naturhistorisches Museum, Wien
SMF   Naturmuseum Senckenberg, Frankfurt am Main
SMNH   Naturhistoriska Riksmuseet, Stockholm

Countries:

A   Austria
AL   Albania
BG   Bulgaria
F   France
GR   Greece
I   Italy
MK   Macedonia
MNE   Montenegro
SK   Slovakia
SLO   Slovenia

Taxonomy

Family Orculidae Steenberg 1925

Genus *Orcula* Held 1837

1837   *Orcula* Held: 919. Type species *Pupa dolium* Draparnaud 1801 (by subsequent designation, Gray 1847: 176).

1852   *Pupula* Mörch: 34 (nom. praeocc., non Charpentier 1837). Type species *Pupa dolium* Draparnaud 1801 (by subsequent designation, Pilsbry 1922: 1).

1907   *Doliana* Caziot: 225. Type species *Pupa* [*Orcula*] *dolium* Draparnaud 1801 (by subsequent designation, Hausdorf 1996: 11).

N o t e s : Caziot divided the former species of the genus *Orcula* into three groups, mainly based on their distribution. The first group, Raymondiana, did not contain any *Orcula* species. The second group, Doliolana, with *Orcula* [*Sphyradium*] *doliolum* as new defined type species, consisted of species of the south-central Taurica, the Alps, and south-central Spain. Doliolana included *Orcula gularis* var. *spoliata* [*O. spoliata*], *Orcula* [*schmidtii*] *transversalis*, and *Orcula* [*dolium*] *uniplicata*. The third group, Doliana, consisted of species of the south-central Alps and included the remaining *Orcula* taxa that had been described until then: *O. conica*, *O. dolium dolium, O. dolium brancsikii*, *O. gularis*, *O. jetschini*, *O. pollonerae*, and *O. schmidtii*. The "group-names" set up by

Caziot should not be considered as valid. The system separates closely related *Orcula*-species into different groups and in Doliolana even combines them with a species of another genus. Furthermore, *Orcula spoliata* and its assumed synonym, *Orcula pollonerae*, were positioned in two different groups. Hausdorf (1996: 13–14) wrote about the "group-names" set up by the "nouvelle école" and explained the reasons why the names must not be used.

Orcula austriaca austriaca Zimmermann 1932
Pl. 1, fig. A

1932   *Orcula spoliata austriaca* Zimmermann: 37.
1978   *Orcula austriaca austriaca*, – Gittenberger: 22, 30–34 (comb. nov.).

T y p e   l o c a l i t y   (label): [A], Niederösterreich, Lilienfeld.

T y p e   m a t e r i a l : Syntypes NHMW 1567/5 (coll. Klemm ex Zimmermann; SH 6.4, SW 2.7, AH 2.0, AW 1.8 mm, 8.5 whorls; pl. 1, fig. A), SMF 54239/5 (coll. Ehrmann ex Zimmermann), SMF 54246/8 (coll. Ehrmann ex Zimmermann), SMF 202728/4 (coll. S.H. Jaeckel ex Zimmermann).

N o t e s : Studies on *O. spoliata* and *O. austriaca* revealed differences in the genital anatomy between the

two taxa that make, according to GITTENBERGER (1978: 22, 30–34), a close relationship implausible.

Orcula austriaca faueri KLEMM 1967
Pl. 1, fig. F

1967 *Orcula faueri* KLEMM: 101.
1978 *Orcula austriaca faueri*, – GITTENBERGER: 30–34 (comb. nov.).

Type locality (label/publication): [A], Kärnten, Karawanken, Hochobirmassiv, Westfuss des Kuhberges, Freibachgraben (ca. 900 m).
Type material: Holotype SMF 188613/1 (coll. ZILCH ex KLEMM, leg. KLEMM, 08.08.1960; SH 6.7, SW 2.9, AH 2.2, AW 1.9 mm, 8.75 whorls); paratypes SMF 188614/6 (coll. ZILCH ex KLEMM, KLEMM leg. 8.8.60), SMF 202706/6 (coll. JAECKEL ex KLEMM 1960), SMF 274703/5 (coll. SCHLICKUM ex KLEMM), NHMW 55624/10 (coll. KLEMM, leg. KLEMM 8.8.1960).

Notes: GITTENBERGER (1978: 30–34) revealed a high similarity between *O. austriaca austriaca* and *O. austriaca faueri* in his anatomical studies and classified *faueri* as subspecies of *O. austriaca*.

Orcula austriaca goelleri GITTENBERGER 1978
Pl. 1, fig. E

1932 *Orcula spoliata austriaca* morpha oreina ZIMMERMANN: 38–39 (nom. nud.).
1978 *Orcula austriaca goelleri*, – GITTENBERGER: 36 (stat. nov. / nom. nov. pro morpha oreina).

Type locality (label): [A], niederösterreichisch-steirische Grenze, [Mt.] Göller (1760 m).
Type material: Syntype LMOB ALT/6685 (coll. ZIMMERMANN; SH 5.2, SW 2.7, AH 1.9, AW 1.7 mm, 8 whorls), SMF 54241/1 (coll. EHRMANN ex ZIMMERMANN).

Notes: ZIMMERMANN's morpha "oreina" of *O. spoliata austriaca* was first an addition to a trinomen, and second described as morpha, therefore the name is not available according to the ICZN (1999: Articles 45.5 and 45.6.2). Furthermore, the former ZIMMERMANN taxa, *O. austriaca* oreina and *O. gularis* oreina, were primary homonyms (ICZN 1999: Article 57.2). GITTENBERGER (1978: 36) gave *O. gularis oreina* the priority and introduced the nomen novum *O. austriaca goelleri* for *O. [spoliata] austriaca* oreina. The name refers to the type locality of the taxon.

Orcula austriaca pseudofuchsi KLEMM 1967
Pl. 1, fig. B

1967 *Orcula pseudofuchsi* KLEMM: 107.
1978 *Orcula austriaca pseudofuchsi*, – GITTENBERGER: 30–34 (comb. nov.).

Type locality (label/publication): [A], Niederösterreich, bei Ternitz, oberste Felswände des Gösingberges (800 m).

Type material: Holotype SMF 188621/1 (coll. ZILCH ex KLEMM, leg. KLEMM, 18.08.1956; SH 7.7, SW 2.9, AH 2.1, AW 1.9 mm, 10 whorls), paratypes SMF 188622/6 (coll. ZILCH ex KLEMM), SMF 274707/6 (coll. SCHLICKUM ex KLEMM), SMF 202721/6 (coll. JAECKEL ex KLEMM), NHMW 55625/8 (coll. KLEMM, leg. KLEMM 18.8.1956), NHMW Orc 153/6 (leg. KLEMM 18.8.1956).

Notes: Anatomical studies of *O. austriaca austriaca* and *O. austriaca pseudofuchsi* by GITTENBERGER (1978: 30–34) revealed a high similarity between both taxa. The morphological and zoogeographical relations were already mentioned by KLEMM (1967: 107–108).

Orcula conica (ROSSMÄSSLER 1837)
Pl. 4, fig. A–B

1837 *Pupa conica* ROSSMÄSSLER: 17.
1837 *Orcula conica*, – HELD: 919.

Type locality (label): [SLO], Kärnten, Krain [Kranjska - formerly part of Austria]. Type locality (publication): Krain [Kranjska], Voralpen um Laibach [Lower Alps near Ljubljana].
Type material: Syntype SMF 5004/1 LT (SH 5.9, SW 3.1, AH 2.0, AW 1.8 mm, 9.5 whorls).

Notes: The specimen SMF 5004/1 LT was probably chosen by A. ZILCH as a possible lectotype for the species (indicated by the labelling "LT") but as this was not published we treat the specimen as a syntype still. A picture of a second syntype (SMF 5005/13) is also shown as this specimen has a better preserved periostracum (SH 6.1, SW 3.3, AH 2.2, AW 2.0; 8.5 whorls).

Orcula conica [minor] ZIMMERMANN 1932
Pl. 3, fig. C

1932 *Orcula conica* forma localis minor ZIMMERMANN: 42 (nom. nud., H. v. GALLENSTEIN in schedis). The name is not available.

Type locality (label): [A], Kärnten, Gurnitzerschlucht bei Sattnitz.
Type material: "Holotype" LMOB ALT/4915/1 (coll. ZIMMERMANN; SH 4.7, SW 2.9, AH 1.8, AW 1.7 mm, 8.5 whorls); "Paratypes" SMF 54172/2 (coll. EHRMANN ex ZIMMERMANN), "paratypes" NHMW 1555/2 (coll. KLEMM, leg ZIMMERMANN).

Notes: The name "minor" is a homonym of *Pupa [Orcula] dolium* var. *minor* MOQUIN-TANDON 1855 and therefore not available (ICZN 1999: Article 57.2). GITTENBERGER (1965: 97) wrote that specimens of *O. conica* that he had found at the type locality of *O. conica* minor were not smaller than those of typical *O. conica* from the "Karawanken" and that he could not observe specimens of *O. conica* minor as described by ZIMMERMANN. Nevertheless, for the purpose of documentation, ZIMMERMANN's type specimen is shown any way.

Orcula dobrogica (GROSSU 1986)

1986   *Sphyradium dobrogicum* GROSSU: 11–12, fig. 4.
2004   *Orcula dobrogicum*, – BANK: http://www.faunaeur.org, version 1.1. (comb. nov.).
2008   *Orcula dobrogica*, – WELTER-SCHULTES: http://www.animalbase.unigoettingen.de.
2011   *Orcula dobrogica*, – BANK: http://www.faunaeur.org, version 2.4.

Type locality (publication): [RO], Dobrogea, département de Tulcea, près du Monastère Cocoş de la Forêt Luncaviţa.
Type material: Holotype Nr. 1178 (from publication; coll. GROSSU); now probably deposited in the collection of the Muséum d'Histoire Naturelle Grigore Antipa in Bukarest.

Notes: BANK (2004) classified the taxon into the genus *Orcula* in the Fauna Europaea Checklist but the entry is not based on literature data. BANK sighted material of the taxon around the turn of the millennium and recognized it as being an *Orcula* species [personal communication]. Since *Orcula* is feminine, according to WELTER-SCHULTES (2008: http://www.animalbase.unigoettingen.de), the name has to be changed to *Orcula dobrogica*. BANK confirmed this comment as the right decision [personal communication, 2010] and changed the name to *Orcula dobrogica* in 2011 (http://www.faunaeur.org version 2.4). We consider the name as valid, although a final classification of the taxon can not be done until further investigations are undertaken and the results are published.

Orcula dolium dolium (DRAPARNAUD 1801)

Pl. 2, fig. A

1801   *Pupa dolium* DRAPARNAUD: 58–59.
1837   *Orcula dolium*, – HELD: 919.

Type locality: not indicated (France – from the publication's title).
Type material: Syntype NHMW 14765/1820.266.1/2 (SH 6.8, SW 3.3, AH 2.5, AW 2.1 mm, 9 whorls).

Notes: GRAY (1847: 176) classified *Orcula dolium* as the type species of the genus *Orcula* HELD. LOCARD (1894: 322) mentioned *Orcula dolium* as *Orcula doliformis*, which is an unjustified emendation.

Orcula dolium brancsikii CLESSIN 1887

Pl. 3, fig. F

1887   *Orcula brancsikii* CLESSIN: 236–237. (*Pupa dolium* var. *elongata* BRANCSIK in schedis).
1887   *Orcula dolium brancsikii*, – BRANCSIK: 81. (comb. nov.).

Type locality (publication): [SK], Oberungarn, Comitate Trencsin [Trenčin], bei Manin [Považská Bystrica, Maninská tiesňava].
Type material: Syntype NHMW J. N. 22075/1 (voucher specimen of CLESSIN's "Molluskenfauna Österreich Ungarns

und der Schweiz", CLESSIN don. 1894; SH 7.5, SW 2.9, AH 2.3, AW 2.0 mm, 10 whorls).

Notes: CLESSIN described *Orcula brancsikii* based on material that was available to him from BRANCSIK. BRANCSIK collected these specimens and deposited them as *Pupa* [*Orcula*] *dolium* var. *kimakowiczi*, forma "curta" and "elongata", in 1885 and sent several specimens to other collectors, most likely also to CLESSIN. By the way, the two formae represent names that are not available according to the ICZN (1999: Article 5.6). According to CLESSIN (1887: 237), BRANCSIK's taxon "*Pupa dolium* var. elongata" [= *Pupa dolium* var. *kimakowiczi* forma elongata] can not be united with *Pupa* [*Orcula*] *dolium* because of the slender form, the peaked curl, and the higher amount of whorls, for which reason CLESSIN considered the taxon as a new species and named it after its discoverer. BRANCSIK (1887: 81, 84) described the taxon as *Pupa* [*Orcula*] *dolium* var. *kimakowiczi* later in 1887 from his own material and pointed out arguments why CLESSIN's *O. brancsikii* should not be considered a separate species but only a subspecies of *O. dolium*. He based his opinion on the occurrence of countless intermediates between the var. *kimakowiczi* and typical *O. dolium* specimens in the investigated area and the observation that sometimes elongate forms with two columellar folds occurred. LOŽEK (1965: 208) and LISICKÝ (1991: 90) referred to *brancsikii* as a subspecies of *O. dolium*. Both authors wrote "*brancsiki*" without a second "*i*" which would be actually the correct notation but not that of the original description. REISCHÜTZ (1995: 30–33) investigated the genital anatomy of three specimens from the type locality. He observed only marginal differences between *O. brancsikii* and *O. dolium* and wrote that the genital anatomy did not help to decide which status the taxon should be assigned to.

Orcula dolium cebratica (WESTERLUND 1887)

Pl. 2, fig. G

1887   *Pupa* (*Orcula*) *dolium* forma *cebratica* WESTERLUND: 84.

Type locality (label/publication): [SK], Jetschin, Berg Cebrat [Ružomberok, Mt. Čebrat].
Type material: Syntypes MNHG Wstld2090 (SH 7.2, SW 3.2, AH 2.4, AW 1.9; 9 whorls), SMNH RM8:38.

Orcula dolium edita EHRMANN 1933

Pl. 2, fig. B

1932   *Orcula dolium* morpha edita ZIMMERMANN: 17 (nom. nud.).
1933   *Orcula dolium* f. *edita*, - EHRMANN: 50 (comb. nov.).

Type locality (label): [A], Niederösterreich, [Mt.] Schneeberg, [gorge] Eng (1000 m).
Type material: Syntypes LMOB ALT/5319/1 (coll. ZIMMERMANN, leg. ZIMMERMANN; SH 6.5, SW 3.1, AH 2.4, AW

2.0 mm, 8 whorls), SMF 50854/4 (coll. EHRMANN ex ZIMMER-MANN), NHMW 1598/4 (coll. KLEMM, leg. ZIMMERMANN 1930).

N o t e s : FALKNER et al (2001: 32) mentioned *O. dolium edita* as "*O. dolium edita* PILSBRY 1934" in the CLECOM-check-list but PILSBRY (1934: 123–125) just presented ZIMMERMANN's table, plate, and a translation of the original text with comments. An intention to adopt the morpha "edita" as a real subspecies is not evident from the publication. Moreover, this was done before by EHRMANN (1933: 50) who first presented the infrasubspecific morpha "edita" (IRCN 1999: Article 45.6.2) as f. [forma] and therefore, according to the IRCN (1999: Article 45.6.4), as a valid subspecies of *O. dolium*.

Orcula dolium globulosa (LOCARD 1880)

1880   *Pupa* (*Orcula*) *dolium* var. *globulosa* LOCARD: 265–266.

T y p e   l o c a l i t y   (publication): [F], Savoy [/Savoie], l'Isère.
T y p e   m a t e r i a l : Several requests to the Muséum national d'histoire naturelle in Paris for information about the whereabouts of the types of *O. dolium major* remained unanswered.

Orcula dolium gracilior ZIMMERMANN 1932
Pl. 2, fig. D

1932   *Orcula dolium* forma localis *gracilior* ZIMMERMANN: 22.

T y p e   l o c a l i t y   (label): [A], Niederösterreich, [Mt.] Semmering, Adlitzgraben.
T y p e   m a t e r i a l : Syntypes LMOB ALT/5343/1 (coll. ZIMMERMANN; SH 6.2, SW 3.1, AH 2.3, AW 1.9 mm, 8.5 whorls), SMF 49943/5 (coll. EHRMANN ex ZIMMERMANN), NHMW 01578/6 (coll. KLEMM, leg. ZIMMERMANN 1930).

Orcula dolium gracilis (HAZAY 1885)

1885   *Pupa dolium* forma *gracilis* HAZAY: 31.

T y p e   l o c a l i t i e s   (publication): [SK], Kotlina-Thal [Banská Bystrica, Zvolenská kotlina], Waldungen der Bélaer [Poprad, woodland of Belianske Tatry], Landoker Kalkalpen [Kežmarok, limestone alps of Landak], im Zdjarer Pass [Poprad, Ždiar pass] und bei Podspady [Poprad, Podspady].
T y p e   m a t e r i a l : Depository of the type specimens could not be determined by the authors.

Orcula dolium implicata CLESSIN 1887

1887   *Orcula dolium* var. *implicata* CLESSIN: 234. (BRANCSIK in schedis).

T y p e   l o c a l i t y   (publication): [SK], Oberungarn im Comitate Trencsin [Trenčin], bei Vratna [Žilina, Malá Fatra, Vrátna].
T y p e   m a t e r i a l : Depository of the type specimens could not be determined by the authors.

N o t e s : CLESSIN described *Orcula dolium implicata* based on material that was available to him from BRANCSIK. BRANCSIK collected these specimens and designated and deposited them as *Pupa* [*Orcula*] *dolium* var. *implicata* in 1885 and sent specimens to other collectors, probably also to CLESSIN. BRANCSIK (1887: 84 and 1890:21) later wrote that the total absence of both columellar folds was a deformation that occurred only in four of thousands specimens which all stem from the Vrátnatal [Vrátna valley]. Probably "*implicata*" was never meant as a subspecies as all other "formae" mentioned in BRANCSIK's publication, such as "obesa,", "cylindrica", and "curta", "biplicata", "uniplicata", and "triplicata" referred to infrasubspecific additions (ICZN 1999: 45.5) to subspecific taxon names which BRANCSIK used to point out the variability of shell forms of the Carpathian *O. dolium* subspecies.

Orcula dolium infima EHRMANN 1933
Pl. 2, fig. H

1932   *Orcula dolium* morpha *infima* ZIMMERMANN: 14 (nom. nud.).
1933   *Orcula dolium* f. *infima*, - EHRMANN: 50 (comb. nov.).

T y p e   l o c a l i t y   (label): [A], Niederösterreich, Kierling bei Klosterneuburg.
T y p e   m a t e r i a l : Syntypes LMOB ALT/5353/1 (coll. ZIMMERMANN; SH 8.8, SW 4.6, AH 3.2, AW 2.7 mm, 10 whorls), SMF 49939/4 (coll. EHRMANN ex ZIMMERMANN).

N o t e s : FALKNER et al (2001: 32) mentioned *O. dolium infima* as *O. dolium infima* PILSBRY 1934 in the CLECOM-check-list, but PILSBRY (1934: 123–125) just presented ZIMMERMANN's table, plates, and a translation of the original text with comments. An intention to adopt the morpha *infima* as a real subspecies is not evident from the publication. KLEMM (1967: 110) considered *Orcula dolium infima* to be a real geographical subspecies because of its isolated distribution and the size of its shell. Obviously, FALKNER (2001: 32) and KLEMM (1967: 110) did not know that the name was already made available by EHRMANN (1933: 50) who first presented the infrasubspecific morpha "infima" (IRCN 1999: Article 45.6.2) as f. [forma] and therefore, according to the IRCN (1999: Article 45.6.4), as a valid subspecies of *O. dolium*.

Orcula dolium kimakowiczi (BRANCSIK 1887)
Pl. 3, figs. D & E

1887   *Pupa dolium* v. *kimakowiczi* BRANCSIK: 84.
1887   *Pupa dolium kimakowiczi* f. curta BRANCSIK: 84, pl. 1, fig. 19 (nom. nud.). The name is not available.
1887   *Pupa dolium kimakowiczi* f. elongata BRANCSIK: 84, pl. 1, fig. 18 (nom. nud., synonym of *Orcula dolium brancsikii* CLESSIN 1887). The name is not available.

T y p e   l o c a l i t y   (label/publication): [SK], Comitate Trencsin [Trenčin], Manin [Považská Bystrica, Maninská tiesňava].

Type material (for *Pupa* [*Orcula*] *dolium kimakowiczi* f. curta): Syntypes NHMW 68412 (5510)/ 27045/8, labelled as "Original"; SH 7.1, SW 3.1, AH 2.2, AW 2.0 mm, 10.25 whorls; pl. 3, fig. D), NHMW Orc 99/2 (coll. RUSNOV ex BRANCSIK), SMF 49918/5 (coll. REINHARDT ex BRANCSIK), SMF 246033/2 (coll. KALTENBACH ex A. MÜLLER ex BRANCSIK).

Type material (for *Pupa* [*Orcula*] *dolium kimakowiczi* f. elongata): Syntypes NHMW 68410 (5440)/ 27045/6 (labelled as "Original"; SH 8.3, SW 3.1, AH 2.5, AW 2.1 mm, 10.25 whorls; pl. 3, fig. E), Syntypes SMF 49915/6 (coll. O.BOETTGER ex BRANCSIK 1885), SMF 4527/5 (coll. KOBELT ex BRANCSIK), SMF 49912/14 (coll. MOELLENDORFF ex BRANCSIK), SMF 246034/4 (coll. C. BOSCH ? ex BRANCSIK).

Note: *Pupa* [*Orcula*] *dolium kimakowiczi* is probably a synonym of *O. dolium brancsikii* CLESSIN (1887: 236–237) because CLESSIN described the latter one earlier, based on specimens that were available to him from BRANCSIK (see: *Orcula dolium brancsikii* CLESSIN 1887, notes). BRANCSIK described two infrasubspecific formae, "curta" and "elongata" that are not available according to the ICZN (1999: Article 45.5). Both formae just represent different shell variations in specimens of *kimakowiczi* that even stem from the same locality. Nevertheless, for the purpose of documentation, data and pictures of both formae are given subsequently.

Orcula dolium major (LOCARD 1880)

1880   *Pupa* (*Orcula*) *dolium* var. *major* LOCARD: 265.

Type locality (publication): [F, Rhône], les environs de Lyon.
Type material: Several requests to the Muséum national d'histoire naturelle in Paris for information about the whereabouts of the types of *O. dolium major* remained unanswered.

Orcula dolium major (WESTERLUND 1887)

1887   *Pupa* (*Orcula*) *dolium* forma major WESTERLUND: 84. The name is not available.

Type locality (publication): Ungarn [Ungarn/Hungary of that time comprises nowadays several Eastern-European countries].
Type material: Depository of the type specimens could not be determined by the authors.

Notes: The name "major" was used as subspecific addition to *Orcula dolium* earlier by LOCARD (1880: 265). WESTERLUND's (1887: 84) forma "major" for *O. dolium* is therefore a primary homonym (ICZN 1999: Article 57.2) and not available.

Orcula dolium minima (BRANCSIK 1887)

Pl. 3, fig. A, B, & C

1887   *Pupa dolium* v. *minima* BRANCSIK: 83.
1887   *Pupa dolium minima* f. cylindrica BRANCSIK: 83, pl. 1, fig. 16 (nom. nud.). The name is not available.

1887   *Pupa dolium minima* f. curta BRANCSIK: 83, pl. 1, fig. 17 (nom. nud.). The name is not available.
1887   *Pupa dolium minima* f. obesa BRANCSIK: 83, pl. 1, fig. 15 (nom. nud.). The name is not available.

Type locality (label/publication): [SK], Trencsin [Trenčín]: Rajecz-Teplicz [Žilina, Rajecké Teplice].
Type material (for *Pupa* [*Orcula*] *dolium minima* f. cylindrica): Syntype NHMW 27044/2 (labelled as "Original", SH 6.0, SW 3.0, AH 2.2, AW 1.9 mm, 8 whorls; pl. 3, fig. A), SMF 5006/2 (coll. KOBELT ex BRANCSIK).
Type material (for *Pupa* [*Orcula*] *dolium minima* f. curta): Syntypes NHMW 27044/2 (labelled as "Original", SH 5.1, SW 2.7, AH 2.0, AW 1.7 mm, 7.5 whorls; pl. 3, fig. B), SMF 49916/2 (coll. O. REINHARDT ex BRANCSIK), SMF 246032/3 (coll. H. KALTENBACH ex A. MÜLLER ex BRANCSIK).
Type material (for *Pupa* [*Orcula*] *dolium minima* f. obesa): Syntypes NHMW 68376/4 (labelled as "Original"; SH 5.6, SW 2.8, AH 2.1, AW 1.8 mm, 8 whorls; pl. 3, fig. C), SMF 5008/4 (coll. O. REINHARDT ex BRANCSIK).

Notes: BRANCSIK described three forms of *O. dolium* var. *minima* which he defined as forma "obesa", "curta", and "cylindrica". These infrasubspecific names just represent different shell variations in specimens of *minima* that even stem from the same locality and are not available according to the ICZN (1999: Art. 45.5.). Nevertheless, for the purpose of documentation, data and pictures of all three formae are given.

Orcula dolium minor (MOQUIN-TANDON 1855)

1855   *Pupa* (*Orcula*) var. *dolium minor* MOQUIN-TANDON: 384.

Type locality (publication): [F, Côte-d'Or], Prés de Dijon.
Type material: Several requests to the Muséum national d'histoire naturelle in Paris for information about the whereabouts of the types of *O. dolium minor* remained unanswered.

Orcula dolium obesa (WESTERLUND 1887)

Pl. 3, fig. I

1887   *Pupa* (*Orcula*) *dolium* forma *obesa* WESTERLUND: 84.

Type locality (publication): Ungarn [Ungarn/Hungary of that time comprises nowadays several Eastern-European countries].
Type material: Syntypes MNHG Wstld2086 (SH 8.3, SW 4.0, AH 2.9, AW 2.4 mm, 8.75 whorls), and SMNH RM8:38, SMF 50844/5 (coll. KOBELT ex BRANCSIK), SMF 110388/2 (coll. JETSCHIN ex BRANCSIK 1887).

Orcula dolium par (WESTERLUND 1887)

Pl. 2, fig. E

1887   *Pupa* (*Orcula*) *dolium* forma *par* WESTERLUND: 84.

Type locality (label): [A], Tirol, Reutte.
Type material: Syntype MNHG Wstld2089 (SH 6.9, SW 3.4, AH 2.5, AW 2.0 mm, 8.75 whorls).

N o t e s : ZIMMERMANN (1932: 23) examined specimens loaned from the original collection of WESTERLUND in Goteborg. He stated that the specimens from the type locality "Reutte, Tirol" did not show any special characters that notably differed from the typical shell form of the nominative form; therefore, the taxon *O. dolium par* should be neglected. The type specimens sighted for this publication differ from the typical *O. dolium dolium* in their more ovate-conical shell form and the yellowish colour. Further investigations are necessary before ZIMMERMANN's opinion can be confirmed.

Orcula dolium pfeifferi (MOQUIN-TANDON 1855)

1855   *Pupa dolium* var. *pfeifferi* MOQUIN-TANDON: 385. Pl. 1, figs. 8–9.

T y p e   l o c a l i t y (publication): [F, Isère], près de Grenoble.

T y p e   m a t e r i a l : Several requests to the Muséum national d'histoire naturelle in Paris for information about the whereabouts of the types of *O. dolium pfeifferi* remained unanswered.

Orcula dolium pseudogularis A. J. WAGNER 1912

Pl. 2, fig. F

1912   *Orcula dolium* forma *pseudogularis* A. J. WAGNER: 252.

T y p e   l o c a l i t y (label): [A, Niederösterreich], Pittental, Ruine [ruin] Türkensturz bei Gleissenfeld.

T y p e   m a t e r i a l : Syntypes NHMW 56158/2 (det. A. J. WAGNER; SH 7.6, SW 3.9, AH 2.8, AW 2.3 mm, whorls 9.0), SMF 110405/12 (coll. JETSCHIN ex WAGNER), SMF 110406/1 (coll. C.R. BOETTGER ex Mus. Warsaw ex coll. WAGNER).

N o t e s : PILSBRY (1922: 11) pointed out the similarity of *O. dolium pseudogularis* and *O. dolium uniplicata*. See: *O. dolium uniplicata* (POTIEZ & MICHAUD 1838). Anyway, a reduction of the upper columellar fold as it was described for both subspecies, is a characteristic that can be found in many populations covering the whole distribution of *O. dolium* and we assume it to be a convergent formation.

Dr. DOMINIKA MIERZWA from the Museum and Institute of Zoology in Warsaw was contacted twice as parts of the WAGNER collection should be housed there but she could not locate any types of the genus *Orcula* in the collection.

Orcula dolium quadriplicata (LOCARD 1880)

1880   *Pupa* (*Orcula*) *dolium* var. *quadri-plicata* LOCARD: 266.

T y p e   l o c a l i t y (publication): [F, Rhône], les environs de Lyon.

T y p e   m a t e r i a l : Several requests to the Muséum national d'histoire naturelle in Paris for information about the whereabouts of the types of *O. dolium quadriplicata* remained unanswered.

Orcula dolium raxae GITTENBERGER 1978

Pl. 2, fig. C

1932   *Orcula dolium* morpha oreina ZIMMERMANN: 37 (nom. nud., A. J. WAGNER in schedis).
1933   *Orcula dolium* f. oreina EHRMANN: 50 (stat. nov.).
1978   *Orcula dolium raxae* GITTENBERGER: 36 (nom. nov.).

T y p e   l o c a l i t y (label): [A], Niederösterreich, [Mt.] Rax, Heukuppe, 2000 m.

T y p e   m a t e r i a l : Syntypes LMOB ALT/5354/1 (coll. ZIMMERMANN; SH 5.7, SW 2.9, AH 2.1, AW 1.7 mm, whorls 7.5), SMF 49948/2 (coll. EHRMANN ex ZIMMERMANN).

N o t e s : The former taxa *O. dolium* oreina and *O. gularis* oreina, both described as morphae by ZIMMERMANN (1932: 37), were not only nomina nuda according to the ICZN (1999: Article 45.6.2) but also primary homonyms (ICZN 1999: Article 57.2). EHRMANN (1933: 50) first mentioned ZIMMERMANN's taxon as *Orcula dolium* f. *oreinos* S. ZIMMERMANN and therefore made the name available (IZCN 1999: Article 45.6.2). He changed the name from "oreina" to "oreinos", but as he referred to ZIMMERMANN, "oreina" was still the right notation. GITTENBERGER (1978: 36) gave *O. gularis oreina* the priority and introduced the nomen novum *O. dolium raxae* for the former *O. dolium* oreina.

Orcula dolium tatrica A. J. WAGNER 1922

1922   *Orcula dolium tatrica* A. J. WAGNER: 121–122; pl. VI, fig. 40.

T y p e   l o c a l i t y (publication): [probably CZ or PL], Tatra.

T y p e   m a t e r i a l : Depository of the type specimens could not be determined by the authors (see: notes).

N o t e s : In the Fauna Europaea checklist (BANK 2004), *Orcula dolium tatrica* is listed as a synonym of *Orcula dolium brancsikii* CLESSIN 1887. We could not find any data in the literature which justified this decision. The type locality of this taxon is only vaguely defined. According to A. J. WAGNER, the specimens were collected by ŚLÓSARSKI in the "Tatra" but without precise indication of the sample locality. Furthermore, the shell height mentioned in the description (SH 5 mm, SW 2 mm), is considerably smaller than that of typical specimens from the type locality of *O. dolium brancsikii*. Dr. DOMINIKA MIERZWA from the Museum and Institute of Zoology in Warsaw was contacted twice as parts of the WAGNER collection should be housed there but she could not locate any types of the genus *Orcula* in the collection.

Orcula dolium titan (BRANCSIK 1887)

Pl. 2, fig. G, H

1887   *Pupa dolium* v. *titan* BRANCSIK: 82.

1887   *Pupa dolium titan* f. cylindrica BRANCSIK: 82, pl. 1, fig. 13 (nom. nud.). The name is not available.

1887   *Pupa dolium titan* f. obesa BRANCSIK: 82, pl. 1, fig. 12 (nom. nud.). The name is not   available.

1887   *Pupa dolium titan* f. curta BRANCSIK: 83, pl. 1, fig. 14 (nom. nud.). The name is not   available.

T y p e   l o c a l i t y   (label): [SK, Žilinský kraj], Trencsin [Trenčin], Trencsén-Teplicz [Trenčianske Teplice], [Mt.] Klepács [Maly Klepač].

Type material (for *Pupa* [*Orcula*] *dolium titan* f. cylindrica): Syntypes NHMW 68377/(5448)/3 (labelled as "Original"; coll. OBERWIMMER, leg. BRANCSIK; SH 7.8, SW 4.0, AH 2.9, AW 2.3 mm, 8.5 whorls; pl. 2, fig. J).

Type material (for *Pupa* [*Orcula*] *dolium titan* f. obesa): Syntypes NHMW 68413/ (5449)/3 (labelled as "Original"; coll. OBERWIMMER, leg. BRANCSIK; SH 8.0, SW 4.2, AH 3.2, AW 2.6 mm, 8.5 whorls; pl. 2, fig. K), Syntype [?] MTC 7713/1 (coll. BRANCSIK) according to GROSSU (1974: 196), SMF 49926/2 (coll. O. REINHARDT ex BRANCSIK).

Type material (for *Pupa* [*Orcula*] *dolium titan* f. curta): Syntypes NHMW 68375/(5511)/3 (labelled as "Original", coll. OBERWIMMER, leg. BRANCSIK; SH 7.6, SW 3.9, AH 2.8, AW 2.3 mm, 8 whorls; picture not shown), SMF 5007/1 (coll. KOBELT ex BRANCSIK).

N o t e s : BRANCSIK described three formae for *Pupa dolium* var. *titan* whom he defined as forma "obesa", "curta", and "cylindrica". The names for these three formae are infrasubspecific and therefore not available (ICZN Art. 45.5). The formae just represent different shell variations in specimens of *titan* that even stem from the same locality. Nevertheless, for the purpose of documentation, data and pictures of all three forms are given subsequently. According to GROSSU (1974: 185–186), the main part of the BRANCSIK collection was housed in the Hungarian National Museum (Magyar Nemzeti Múzeum) in Budapest.

Orcula dolium triplicata CLESSIN 1887

1887   *Orcula dolium* var. *triplicata* CLESSIN: 235–236 (BRANCSIK in schedis).

T y p e   l o c a l i t y   (publication): [SK], Oberungarn, bei Trencsin-Teplitz [Žilina, Trenčianske Teplice].

T y p e   m a t e r i a l : Depository of the type specimens could not be determined by the authors.

N o t e s : CLESSIN (1887: 235–236) described *Orcula dolium triplicata* based on material that was available to him from BRANCSIK. BRANCSIK collected these specimens and designated and deposited them as *Pupa* [*Orcula*] *dolium* var. *triplicata* in 1885. He sent several specimens to friends and collectors, probably also to CLESSIN. BRANCSIK (1887: 84) wrote, that he noticed a formation of a third columellar fold only in single specimens of *O. dolium titan* and the common *O. dolium dolium*. Probably BRANCSIK's "triplicata" was not meant as a subspecies as all other "formae" mentioned in BRANCSIK's publica-

tion, such as "obesa,", "cylindrica", "curta", "biplicata", "uniplicata", and "implicata" referred to infrasubspecific additions (ICZN 1999: 45.5) to subspecific taxon names which he used to point out the variability of shell formations of the Carpathian *O. dolium* subspecies.

Orcula dolium tumida (HAZAY 1885)

1885   *Pupa dolium* forma *tumida* HAZAY: 31.

Type localities (publication): [SK], Kotlina-Thal [Banská Bystrica, Zvolenská kotlina], Waldungen der Bélaer [Poprad, woodland of Belianske Tatry], Landoker Kalkalpen [Kežmarok, limestone alps of Landak], im Zdjarer Pass [Poprad, Ždiar pass] und bei Podspady [Poprad, Podspady].

T y p e   m a t e r i a l : Depository of type specimens could not be determined by the authors.

Orcula dolium uniplicata (POTIEZ & MICHAUD 1838)

1838   *Pupa uniplicata* POTIEZ & MICHAUD: 176; pl. 17, fig. 13–14 (ZIEGL. ex fide ipsâ).

1922   *Orcula dolium uniplicata*, – PILSBRY: 10 (comb. nov.).

T y p e   l o c a l i t y   (publication): [A], les hautes montagnes des Alpes Autrichiennes [Austrian Alps].

T y p e   m a t e r i a l : Several requests to the Muséum national d'histoire naturelle in Paris for information about the whereabouts of the types of *O. dolium unipli*cata remained unanswered.

Orcula fuchsi ZIMMERMANN 1931

Pl. 1, fig. A

1931   *Orcula fuchsi* ZIMMERMANN: 44. Tafel VI, fig. 3–5.

T y p e   l o c a l i t y   (label): [A], Niederösterreich, Turmmauer bei Kernhof.

T y p e   m a t e r i a l : Syntypes LMOB ALT/5596/2 (coll. ZIMERMANN; SH 7.5, SW 2.7, AH 2.1, AW 1.6 mm, 9.0 whorls), NHMW Orc117 (coll. RUSNOV, per KÄUFEL, leg. A. FUCHS), NHMW 51871 (coll. KLEMM, leg. FUCHS).

Orcula gularis gularis (ROSSMÄSSLER 1837)

Pl. 1, fig. I–J

1837   *Pupa gularis* ROSSMÄSSLER: 17–18.

1837   *Orcula gularis*, – HELD: 919.

T y p e   l o c a l i t y   (label/publication): [A], Kärnten, auf dem Loibl [on the Loibl pass].

T y p e   m a t e r i a l : Syntypes SMF 4531/1 [JC 333] (labelled as "LT"; SH 6.1, SW 2.8, AH 2.1, AW 1.7 mm, 8.25 whorls; pl. 1, fig. I), SMF 4532/1 (SH 6.1, SW 2.7, AH 1.8, AW 1.6 mm, 8.5 whorls; pl. 1, fig. J).

N o t e s : The first specimen shown was probably chosen by ZILCH as a Lectotype for the species but as he did not publish that, we consider the specimen still a syntype. A picture of a second syntype (SMF 4532/1) is also shown as this specimen shows a better preserved periostracum.

Orcula gularis oreina Ehrmann 1933
Pl. 1, fig. J

1932   *Orcula gularis* morpha oreina Zimmermann: 30 (nom. nud.).
1933   *Orcula gularis* f. *oreinos* Ehrmann: 50 (stat. nov.).

Type locality (label): [A, Steiermark], Gesäuse, [Mt.] Großer Buchstein (1700–1800 m).
Type material: Holotype LMOB ALT/5703/1 (coll. Zimmermann; SH 5.6, SW 2.5, AH 1.9, AW 1.7 mm, 8.5 whorls), paratypes NHMW 01595/2 (coll. Klemm ex. Zimmermann), NHMW 01565/1 (coll. Klemm leg. Zimmermann), SMF 54219/2 (coll. Ehrmann ex Zimmermann).

Notes: The former taxa *O. dolium oreina* and *O. gularis oreina*, both described as morphae by Zimmermann (1932: 37), were not only nomina nuda according to the ICZN (1999: Article 45.6.2) but also homonyms (ICZN 1999: Article 57.2.3). Ehrmann (1933: 50) first mentioned Zimmermann's taxon as *Orcula gularis* f. *oreinos* S. Zimmermann and therefore made the name available (IZCN 1999: Article 45.6.2). He changed the name from "oreina" to "oreinos", but as he referred to Zimmermann, "oreina" was still the right notation. However, the taxon was still a homonym because Ehrmann applied the subspecific denotation "oreinos" also to Zimmermann's *Orcula dolium* oreina. Gittenberger (1978: 36) gave *O. gularis oreina* the priority and therefore the name is available from Ehrmann (1933: 50). Falkner et al. (2001: 32) mentioned the taxon as *O. gularis oreina* Pilsbry 1934 in the CLECOM-Check-list. Pilsbry (1934: 123–129) just presented Zimmermann's table, plate, and a translation of the original text with comments. An intention to adopt the morpha *oreina* as a real geographical subspecies is not evident from the publication and would not have any consequences.

Orcula gularis reducta Zimmermann 1932
Pl. 1, fig. H

1932   *Orcula gulari*s abberatio reducta Zimmermann: 31–32 (nom. nud.). The name is not available.

Type locality (label): [A], Osttirol, Nordwesthänge des [Mt.] Spitzkofel bei Lienz (1700 m).
Type material: "Holotype" LMOB ALT/5704/1 (coll. Zimmermann; SH 7.1, SW 3.1, AH 2.2, AW 1.9 mm, 9 whorls), "paratypes" SMF 54218/2 (coll. Ehrmann ex Zimmermann).

Notes: The name *Orcula gularis* reducta is not valid according to the ICZN as Zimmermann used the term "aberratio" and *O. gularis* reducta was not adopted as the valid name for a subspecies (ICZN 1999: Articles 45.6.2 and 45.6.4). For the purpose of documentation, we present the specimen deposited by Zimmermann anyhow.

Orcula jetschini (Kimakowicz 1883)
Pl. 4, fig. J

1883   *Pupa* (*Orcula*) *jetschini* Kimakowicz: 34–35.

Type localities (publication): [RO], SW Siebenbürgen [Transilvania], Vajda-Hunyad [Hunedoara/ Vajdahunyad]; Banat, Cernathal [Valea Cernei] bei Mehadia; Zalatna, Judenberg [Zsidóhegy];
Type material: Syntype [?] NHMW Orc. 149/1 (labelled as "Co-type"; [RO], Transilvania, Cluj-Napoca; coll. Rusnov, ex coll. Dr. W. Blume; SH 6.1, SW 3.7, AH 2.2, AW 1.9 mm, 8.25 whorls).

Notes: The specimen shown is labelled as "Co-type" but the locality is not one of the type localities mentioned in the publication. Therefore, we are not sure if it is really a type specimen.

Orcula pseudodolium A. J. Wagner 1912
Pl. 1, fig. C

1912   *Orcula gularis* forma *pseudodolium* A. J. Wagner: 252.
1978   *Orcula pseudodolium*, – Gittenberger: 26–27 (stat. nov.).

Type locality (publication/label): [A], Oberösterreich, Hochsengsengebirge bei Windischgarsten, Feuchtenaueralm.
Type material: Syntype LMOB ALT/2018/1 (leg. A. J. Wagner; SH 7.3, SW 3.3, AH 2.6, AW 2.0 mm, 9 whorls), NHMW 1576/4 (coll. Klemm ex Wagner), NHMW 56160/1 (det. A.J. Wagner).

Notes: *O. pseudodolium* often was treated as an intermediate form between *O. dolium* and *O. gularis* (e.g. Zimmermann 1932: 24, Klemm 1974: 132, Pilsbry 1934: 128–129) because it shares shell characters with both taxa. Gittenberger (1978: 26–27) noted that even anatomical studies did not reveal the taxonomical status of the taxon and treated it as a discrete species *Orcula pseudodolium*. He wrote that it can not simply be regarded as a link between *O. dolium* and *O. gularis* and generally can be distinguished from the other two species. Dr. Dominika Mierzwa from the Museum and Institute of Zoology in Warsaw was contacted twice as parts of the Wagner collection should be housed there but she could not locate any types of the genus *Orcula* in the collection.

Orcula restituta (Westerlund 1887)
Pl. 4, fig. D

1887   *Pupa* (*Orcula*) *gularis* forma *restituta* Westerlund: 85.
1967   *Orcula restituta*, – Klemm: 104 (stat. nov.).

Type locality (label): [SLO], Krain, Feistritzthal bei Stein [Kranjska, Kamnik, Kamniška Bistrica valley]: Robič.
Type material: Syntype MNHG Wstld2101 (SH 6.3, SW 2.7, AH 2.0, AW 1.6 mm, 9 whorls), SMNH (RM8:39).

Notes: Klemm (1967: 104) mentioned the dissimilarity between *O. gularis* and *O. restituta*, and wrote that

the shell characters of the latter one are more similar to those of *O. conica*. Therefore, he treated *Orcula restituta* as a separate species.

Orcula schmidtii schmidtii (KÜSTER 1843 ?)

Pl. 4, fig. H

1843   *Pupa schmidtii* KÜSTER (in MARTINI & CHEMNITZ): 26, pl. 3, fig. 20.

T y p e   l o c a l i t y   (publication): [MNE] Montenegro, Cetinje, unter Steinen auf Kalkfelsen [under stones, on limestone rocks].
T y p e   m a t e r i a l : Depository of the type specimens could not be determined by the authors.

N o t e s : A picture of one specimen from the type locality (Montenegro, Cetinje) is shown. NHMW 68433/3 (coll. OBERWIMMER, det. STURANY; SH 5.2, SW 3.0, AH 1.8, AW 1.9 mm, 8.5 whorls). GITTENBERGER (1978: 36) wrote that according to SMITH & ENGLAND (1937: 92), KÜSTER's description of *O. schmidtii* was published not later than in 1843 and not before 1841, so the date of description "1859", mentioned by JAECKEL, KLEMM & MEISE (1958: 147), is based on an error.

Orcula schmidtii transversalis (WESTERLUND 1894)

Pl. 4, fig. I

1894   *Pupa (Orcula) transversalis* WESTERLUND: 171–172 (pro forte variatio *P. dolii* Drp.).
1987   *Orcula schmidtii transversalis*, – HAUSDORF: 51–53 (stat. nov.).

T y p e   l o c a l i t y   (publication): [GR] Griechenland bei Tschumerka [Athamaniko/Tzoumerka Mountains] im M. Pindos [Pindos Mountains].
T y p e   m a t e r i a l : According to HAUSDORF (1987: 55), the holotype is in the WESTERLUND collection in the Naturhistoriska Museet Göteborg (MNHG).

N o t e s : HAUSDORF (1987: 51–53) stated that *transversalis* stands so close to *Orcula schmidtii* in its shell morphology that it preliminary can be assigned as a subspecies to the latter one.
As the type specimens were not available we show a picture of a specimen, NHMW 102.707 (leg. SATTMANN 1988, det. REISCHÜTZ; SH 6.1, SW 3.4, AH 2.2, AW 2.0, 8.75 whorls), from [GR] Griechenland, [Pindos mountains], Epirus, road Papingo to [Mt.] Timpfi, Geröllhalde [rocky slope].

Orcula spoliata (ROSSMÄSSLER 1837)

Pl. 4, fig. E

1837   *Pupa gularis* var. *spoliata* ROSSMÄSSLER: 18.
1932   *Orcula spoliata*, – ZIMMERMANN: 35–36 (stat. nov.).

T y p e   l o c a l i t y   (publication): [I], Tiroler Alpen [Südtirol/Alto Adige, Alps]. See also: Notes.

T y p e   m a t e r i a l : Type specimens were deposited in SMF but are missing [personal communication of RONALD JANSSEN].

N o t e s : GREDLER (1856: 82) assumed that the places mentioned in his publication were the original type localities as the type specimens of *O. spoliata* had been found by STENTZ jun. (also mentioned by ROSSMÄSSLER, 1837: 18) in the following two places: [I, Südtirol/Alto Adige], Seis [Seis am Schlern/Siusi allo Sciliar] und Kastelrut [Kastelruth/Castelrotto].
According to ZIMMERMANN (1932: 35–36), the shells of *Orcula gularis* and *Orcula spoliata* differed in many characters, and therefore both taxa could be regarded as different species.
PINI (1884: 79) described a new species, *Pupa* [*Orcula*] *pollonerae*, with specimens from the "Valle di Non" which GREDLER (1894: 18) considered to belong to *Orcula spoliata* (see: *Orcula pollonerae*). The name *Orcula pollonerae* is still available, but the status as an independent taxon other than a synonym of *Orcula spoliata* is very unlikely as the description of *Orcula pollonerae* fits perfectly on what is commonly considered to be *Orcula spoliata*. A major problem that complicates a final decision is that ROSSMÄSSLER's description of *Orcula spoliata* is very sparse and collection material from the type localities could not be located for this paper. As mentioned above, the type material was located in the SMF collection but got lost. In the NHMW's and SMF's collections specimens designated as *Orcula spoliata* are only available from Fennberg [Favogna], Südtirol [Alto Adige]. One of the specimens is shown here. NHMW 5820/1 (coll. EDLAUER, leg. ZIMMERMANN; "[I], Südtirol [/Alto Adige], Fennberg [/Favogna]"; SH 6.6, SW 3.0, AH 2.2, AW 2.1 mm, 8.5 whorls; pl. 3, fig. H).

Orcula pollonerae (PINI 1884)

1884   *Pupa pollonerae* PINI: 79.

T y p e   l o c a l i t y   (publication): Tirolia [I, Trentino-Alto Adige], Valle di Non.
T y p e   m a t e r i a l : Depository of the type specimens is probably in the Museo civico di storia naturale in Milano. At least the museum's homepage mentioned the whereabouts of the PINI collection in Milano. Anyhow, our request for the collection numbers remained unanswered.

N o t e s : *Orcula pollonerae* is an available name but it is commonly considered to be a synonym of *O. spoliata* in literature (ZIMMERMANN 1932: 35–36). Most taxonomists who dealt with the genus, such as PILSBRY, KLEMM, or GITTENBERGER did not even mention the taxon. GREDLER (1894: 18) considered *Pupa pollonerae* to be a synonym of *Pupa gularis* var. *spoliata* [now *Orcula spoliata*]. He stated that PINI probably described the taxon in lack of knowledge that it already had been described by ROSSMÄSSLER in 1837 (:18), and that *Orcula spoliata* already had been found in the "Valle di Non" before PINI

described *Pupa* [*Orcula*] *pollone*rae. See: *Orcula spoliata*, notes.

Orcula tolminensis A. J. WAGNER 1912

Pl. 1, fig. D

1912   *Orcula gularis* forma *tolminensis* WAGNER: 253.
1932   *Orcula tolminensis*, – ZIMMERMANN: 33–34 (stat. nov.).

Type locality (label): [SLO], Tolmein [Tolmin], Isonzotal [Isonzo valley], Wasserfall Peričnik [Peričnik falls]. See also: Notes.

Type material: Syntypes LMOB ALT/6799 (coll. ZIMMERMANN ex A. J. WAGNER; SH 5.2, SW 2.5, AH 1.8, AW 1.6 mm, 8 whorls), NHMW 1593/7 (coll. KLEMM ex A. J. WAGNER), NHMW 56159/1 (A. J. WAGNER det.; labelled as "Co-type").

Notes: According to ZIMMERMANN (1932: 33–34), *tolminensis* had to be considered a good species because of its characters differing rather strongly from *gularis*. ZIMMERMANN also mentioned that the Peričnik falls were situated in another locality. Type locality revised: [SLO] im Vranatale [Vrana valley] am Nordabhang [north side] des Triglav, Julische Alpen [Julijske Alpe], Wasserfall Pericnik [Peričnik falls].

Orcula wagneri wagneri STURANY 1914

Pl. 4, fig. A, B, C, D, E, F, G & J

1914   *Orcula wagneri* STURANY in STURANY & WAGNER: 45, pl. 15, fig. 82 a–b.

Type locality Holotype (label/publication): Albanien [AL], Mirdita [Mirditë], Berg [Mt.] Munela bei Oroshi [Orosh].

Type material: Holotype NHMW 41175/1 (leg. BULJABAŠIĆ 1905; SH 6.7, SW 3.3, AH 2.3, AW 2.0 mm, 9 whorls; pl. 4, fig. A). — Paratypes NHMW 41186/2 (leg. BULJABAŠIĆ, 1905; SH 6.3, SW 3.3, AH 2.4, AW 2.2 mm, 7.75 whorls; pl. 4, fig. B). Type locality (label/publication): Albanien [AL], Mirdita [Mirditë], Zebia [Mali i Zebës] bei Oroshi [Orosh].; Paratypes NHMW 43916/1 (leg. WINNEGUTH, 1906; SH 5.5, SW 3.3, AH 2.1, AW 2.1 mm, 7.25 whorls; pl. 4, fig. C). Type locality (label/publication): Albanien [AL], Mirdita [Mirditë], Berg Munela [Mt. Munela] bei Oroshi [Orosh].; Paratypes NHMW 41234/1 (leg. BULJABAŠIĆ; SH 5.5, SW 3.0, AH 2.1, AW 2.0 mm, 7.25 whorls; pl. 4, fig. D). Type locality (label/publication): [AL, Kukës], Ljuma-Gebirge [Ljuma mountains], am Drin [besides the Drin river], unterhalb des Koritnik-Gebirges [below the Koritnik mountains].; Paratypes NHMW 41235/1 (leg. BULJABAŠIĆ, 1904; SH 4.4, SW 2.8, AH 1.7, AW 1.7 mm, 8 whorls; pl. 4, fig. E). Type locality (label/publication): [AL, Mirditë], N. Albanien, Malšent bei Oroshi [Orosh, Mal i Shêit].; Paratypes NHMW 41236/5 (leg. BULJABAŠIĆ, 1904; SH 5.5, SW 3.0, AH 2.1, AW 1.9 mm, 7.25 whorls; pl. 4, fig. F). Type locality (label/publication): [AL, Kukës], N. Albanien, Ljumagebiet [Ljuma mountains], Koritnik-Gebirge [Koritnik mountains], ca. 2000 m.; Paratypes NHMW 43917/3 (leg. WINNEGUTH, 1906; SH 5.7, SW 2.8, AH 2.1, AW 1.8 mm, 8.25 whorls; pl. 4, fig. G). Type locality (label/publication): Albanien [AL], Mirdita [Mirditë], Berg Zebja [Mali i Zebës] bei Oroshi [Orosh].; Paratypes ? NHMW 68431/9 [labelled as "original", see: Notes] (leg. BULJABAŠIĆ, 1904; SH 6.7, SW 3.1, AH 2.4, AW 2.0 mm, 7.75 whorls; pl. 4, fig. H.). Type locality (label): [AL], Alban. montenegr. Grenze [Albanian-Kosovarien border], Baštrik [Pashtrik], circa 2700m.

Notes: The NHMW collection of the *Orcula wagneri* types consists of the holotype and several paratypes from different localities, also labelled as "original" specimens. These specimens' appearances differ from the shell of the holotype to some extent, so we think it is important to show them as well.

The collection numbers of the lots are also indicated by handwriting in the exemplar of STURANY's publication kept in the library of the NHMW, probably written by STURANY himself. Only the lot NHMW 68431/9 is not indicated by handwriting.

Orcula wagneri ljubetenensis STURANY 1914

Pl. 4, fig. I & J

1914   *Orcula wagneri ljubetenensis* STURANY in STURANY & WAGNER: 46, pl. 14, fig. 81.

Type locality (label): [MK, Macedonian-Kosovarian border], Schar Dagh [Šar planina], Ljubeten [Mt. Ljuboten], nordwestlich von Üsküb [north-west from Skopje].

Type material: Syntype NHMW 44422/9 (leg. APFELBECK und ATTEMS 1906; SH 6.0, SW 3.0, AH 2.3, AW 2.1 mm, 7.5 whorls; pl. 4, fig. I).

Notes: The collection number of the lot of the first specimen shown in plate 5 (NHMW 44422) is indicated by handwriting in the exemplar of STURANY's publication kept in the library of the NHMW, probably written by STURANY himself. Another specimen, labeled as "Original" but not indicated in the publication by handwriting, is also shown. NHMW 68432/6 (leg. APFELBECK und ATTEMS; coll. OBERWIMMER; SH 6.1, SW 3.0, AH 2.1, AW 1.8; 8 whorls; pl. 4, fig. J).

Orcula zilchi URBAŃSKI 1960

Pl. 4, fig. F & G

1960   *Orcula zilchi* URBAŃSKI: 57–60.

Type locality (publication): [BG], Nordostbulgarien, etwa 30 km südlich bzw. südöstlich von Burgas [ca. 30 km south or south/south-east of Bourgas], am rechten Ufer des Ropotamo, etwa 3 km vor seiner Mündung [right side of the river Ropotamo, about 3 km upstream of its estuary].

Type material: Holotype Nr. 102/1 (from publication; coll. URBAŃSKI, probably in the Warsaw Museum); paratypes Nr. 103/12 (from publication; coll. URBAŃSKI, probably in the Warsaw Museum), SMF 172902/2 (coll. ZILCH ex URBANSKI 10.1958).

Notes: We missed to loan the paratypes from the SMF and our requests for types from the Warsaw museum

were not successful. Therefore, we show two specimens from Turkey, collected by BARNA PÁLL-GERGELY (2010: 91). *O. zilchi* specimen no. 1 is from "TR, [Bilecik], between Bozüyük and Inegöl, by the "Mezit 7" bridge, 580 m"; leg. et det.: PÁLL-GERGELY, 2007.09.30; SH 7.4, SW 3.6, AH 2.5, AW 2.1, 8.5 whorls; pl. 4, fig. F. *O. zilchi* specimen no. 2 is from "TR, Vil. Bolu, Abant Gölü N., 1030 m"; leg. et det.: PÁLL-GERGELY, 2006.05.17; SH 6.5, SW 3.5, AH 2.3, AW 2.0; 8 whorls; pl. 4, fig. G.

Acknowledgements

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List of the taxa

The following list contains the names of all taxa described until now, presented in alphabetical order. Names which are available according to the ICZN (1999) are written in italics. Names in bold letters are considered as valid by FALKNER et al. (2001) and therefore are present in the CLECOM I-list. If a picture is shown, the type status of the specimen is indicated next to the taxon name. When types of taxa are shown, whose names are not available, this is indicated by quotation marks. Some pictures do not show type specimens, and are marked as "picture shows not type" in the list.

austriaca ZIMMERMANN 1932, *Orcula austriaca* - Syntype

brancsikii CLESSIN 1887, *Orcula dolium* - Syntype ?

cebratica (WESTERLUND 1887), *Orcula dolium* - Syntype

conica (ROSSMÄSSLER 1837), *Orcula conica* - Syntypes

curta (BRANCSIK 1890), *Orcula dolium kimakowiczi* (not available) - "Syntype"

curta (BRANCSIK 1890), *Orcula dolium minima* (not available) - "Syntype"

curta (BRANCSIK 1890), *Orcula dolium titan* (not available) - "Syntype"

cylindrica (BRANCSIK 1890), *Orcula dolium minima* (not available) - "Syntype"

cylindrica (BRANCSIK 1890), *Orcula dolium titan* (not available) - "Syntype"

dobrogica (GROSSU 1986), *Orcula* -

dolium (DRAPARNAUD 1801), *Orcula dolium* - Syntype

edita EHRMANN 1933, *Orcula dolium* - Syntype

elongata (BRANCSIK 1890), *Orcula dolium kimakowiczi* - (not available) "Syntype"

faueri KLEMM 1967, *Orcula austriaca* - Holotype

fuchsi ZIMMERMANN 1931, *Orcula* - Syntype

globulosa (LOCARD 1880), *Orcula dolium* -

goelleri GITTENBERGER 1978, *Orcula austriaca* - Syntype

gracilior ZIMMERMANN 1932, *Orcula dolium* - Syntype

gracilis (HAZAY 1885), *Orcula dolium* -

gularis (ROSSMÄSSLER 1837), *Orcula gularis* - Syntypes

implicata CLESSIN 1887, *Orcula dolium* -

infima EHRMANN 1933, *Orcula dolium* - Syntype

jetschini (KIMAKOWICZ 1883), *Orcula* - Syntype ?

kimakowiczi BRANCSIK 1887, *Orcula dolium* - Syntypes

ljubetenensis STURANY 1914, *Orcula wagneri* - Syntypes

major (LOCARD 1880), *Orcula dolium* -

major (WESTERLUND 1887), *Orcula dolium* - (not available)

minima (BRANCSIK 1890), *Orcula dolium* - Syntypes

minor (MOQUIN-TANDON 1855), *Orcula dolium* -

minor ZIMMERMANN 1932, *Orcula conica* - (not available) "Holotype"

obesa (WESTERLUND 1887), *Orcula dolium* - Syntype

obesa (BRANCSIK 1890), *Orcula dolium minima* - (not available) "Syntype"

obesa (BRANCSIK 1890), *Orcula dolium titan* - (not available) "Syntype"

oreina EHRMANN 1933, *Orcula gularis* - Holotype

par (WESTERLUND 1887), *Orcula dolium* - Syntype

pfeifferi (MOQUIN-TANDON 1855), *Orcula dolium* -

pollonerae (PINI 1884), *Orcula* -

pseudodolium A. J. WAGNER 1912, *Orcula* - Syntype

pseudofuchsi KLEMM 1967, *Orcula austriaca* - Holotype

pseudogularis A. J. WAGNER 1912, *Orcula dolium* - Syntype

quadriplicata (LOCARD 1880), *Orcula dolium* -

raxae GITTENBERGER 1978, *Orcula dolium* - Syntype

reducta ZIMMERMANN 1932, *Orcula gularis* (not available) - "Holotype"

restituta (WESTERLUND 1887), *Orcula* - Syntype

schmidtii (KÜSTER 1843 ?), *Orcula schmidtii* - picture shows no type

spoliata (ROSSMÄSSLER 1837), *Orcula* - picture shows no type

tatrica A. J. WAGNER 1922, *Orcula dolium* -

titan (BRANCSIK 1890), *Orcula dolium* - Syntypes

tolminensis A. J. WAGNER 1912, *Orcula* - Syntype

transversalis (WESTERLUND 1894), *Orcula schmidtii* - picture shows no type

triplicata CLESSIN 1887, *Orcula dolium* -

tumida (HAZAY 1885), *Orcula dolium* -

uniplicata (POTIEZ & MICHAUD 1838), *Orcula dolium* -

wagneri STURANY 1914, *Orcula wagneri* - Holotype & 7 Paratypes

zilchi (URBAŃSKI 1960), *Orcula* - pictures show no type

Plate 1
Pictures Natural History Museum of Vienna (J. Harl)

Fig. A.   *Orcula fuchsi* Zimmermann 1931.
[A], Niederösterreich, Turmmauer bei Kernhof; **Syntype** LMOB ALT/5596/2. Measures: SH 7.5, SW 2.7, AH 2.1, AW 1.6 mm; 9.0 whorls.

Fig. B   *Orcula austriaca pseudofuchsi* Klemm 1967.
[A], Niederösterreich, bei Ternitz, oberste Felswände des Gösingberges (800 m); **Holotype** SMF 188621/1. Measures: SH 7.7, SW 2.9, AH 2.1, AW 1.9 mm; 10 whorls.

Fig. C.   *Orcula pseudodolium* A. J. Wagner 1912.
[A], Oberösterreich, Hochsengsengebirge bei Windischgarsten, Feuchtenaueralm; **Syntype** LMOB ALT/2018/1. Measures: SH 7.3, SW 3.3, AH 2.6, AW 2.0 mm; 9 whorls.

Fig. D.   *Orcula tolminensis* A. J. Wagner 1912.
[SLO], Tolmein [Tolmin], Isonzotal [Isonzo valley], Wasserfall Peričnik [Peričnik falls]; **Syntype** ALT/6799. Measures: SH 5.2, SW 2.5, AH 1.8, AW 1.6 mm; 8 whorls.

Fig. E.   *Orcula austriaca goelleri* Gittenberger 1978.
[A], niederösterreichisch-steirische Grenze, [Mt.] Göller (1760 m); **Syntype**. LMOB ALT/6685. Measures: SH 5.2, SW 2.7, AH 1.9, AW 1.7 mm; 8 whorls.

Fig. F.   *Orcula austriaca faueri* Klemm 1967.
[A], Kärnten, Karawanken, Hochobirmassiv, Westfuss des Kuhberges, Freibachgraben (ca. 900 m); **Holotype** SMF 188613/1. Measures: SH 6.7, SW 2.9, AH 2.2, AW 1.9 mm; 8.75 whorls.

Fig. G.   *Orcula austriaca austriaca* Zimmermann 1932.
[A], Niederösterreich, Lilienfeld; **Syntype** NHMW 1567/5. Measures: SH 6.4, SW 2.7, AH 2.0, AW 1.8 mm; 8.5 whorls.

Fig. H.   *Orcula gularis* reducta Zimmermann 1932.
[A], Osttirol, Nordwesthänge des [Mt.] Spitzkofel bei Lienz (1700 m); **"Holotype"** LMOB ALT/5704/1. Measures: SH 7.1, SW 3.1, AH 2.2, AW 1.9 mm; 9 whorls.

Fig. I-J.   *Orcula gularis gularis* (Rossmässler 1837).
[A], Kärnten, auf dem Loibl [on the Loibl pass]; I.) **Syntype** SMF 4531/1. Measures: SH 6.1, SW 2.8, AH 2.1, AW 1.7 mm; 8.25 whorls; J.) **Syntype** SMF 4532/1. Measures: SH 6.1, SW 2.7, AH 1.8, AW 1.6 mm; 8.5 whorls.

Fig. K.   *Orcula gularis oreina* Zimmermann 1932.
[A, Steiermark], Gesäuse, [Mt.] Großer Buchstein (1700-1800 m); **Holotype** LMOB ALT/5703/1. Measures: SH 5.6, SW 2.5, AH 1.9, AW 1.7 mm; 8.5 whorls.

Plate 1

Plate 2
Pictures Natural History Museum of Vienna (J. HARL)

Fig. A.   *Orcula dolium dolium* (DRAPARNAUD 1801).
France – from the publication's title; **Syntype** NHMW
14765/1820.26.61/2; Measures: SH 6.8, SW 3.3, AH 2.5, AW 2.1
mm; 9 whorls.

Fig. B.   *Orcula dolium edita* EHRMANN 1933.
[A], Niederösterreich, [Mt.] Schneeberg, [gorge] Eng (1000 m);
Syntype LML ALT/5319/1. Measures: SH 6.5, SW 3.1, AH 2.4, AW
2.0 mm; 8 whorls.

Fig. C.   *Orcula dolium raxae* GITTENBERGER 1978.
[A], Niederösterreich, [Mt.] Rax, Heukuppe, 2000 m; **Syntype**
LML ALT/5354/1. Measures: SH 5.7, SW 2.9, AH 2.1, AW 1.7 mm;
whorls 7.5.

Fig. D.   *Orcula dolium gracilior* ZIMMERMANN 1932.
[A], Niederösterreich, [Mt.] Semmering, Adlitzgraben; **Syntype**
LML ALT/5343. Measures: SH 6.2, SW 3.1, AH 2.3, AW 1.9 mm;
8.5 whorls.

Fig. E.   *Orcula dolium par* (WESTERLUND 1887).
[A], Tirol, Reutte; **Syntype** MNHG Wstld2089. Measures: SH 6.9,
SW 3.4, AH 2.5, AW 2.0 mm; 8.75 whorls.

Fig. F.   *Orcula dolium pseudogularis* A. J. WAGNER 1912.
[A, Niederösterreich], Pittental, Ruine [ruin] Türkensturz bei
Gleissenfeld; **Syntype** NHMW 56158. Measures: SH 7.6, SW 3.9,
AH 2.8, AW 2.3 mm; whorls 9.0.

Fig. G.   *Orcula dolium cebratica* (WESTERLUND 1887).
[SK], Jetschin, Berg Cebrat [Ružomberok, Mt. Čebrat]; **Syntype**
MNHG Wstld2090. Measures: SH 7.2, SW 3.2, AH 2.4, AW 1.9; 9
whorls.

Fig. H.   *Orcula dolium infima* EHRMANN 1933.
[A], Niederösterreich, Kierling bei Klosterneuburg; **Syntype** LML
ALT/5353/1. Measures: SH 8.8, SW 4.6, AH 3.2, AW 2.7 mm; 10
whorls.

Plate 2

Plate 3
Pictures Natural History Museum of Vienna (J. Harl)

Fig. A-C.   *Orcula dolium minima* (Brancsik 1890).
[SK], Trencsin [Trenčsin]: Rajecz-Teplicz [Žilina, Rajecké Teplice]:
A.) *Pupa dolium minima* f. cylindrica. "Syntype" NHMW 27044/2
[labelled as "Original"]. Measures: SH 6.0, SW 3.0 , AH 2.2, AW
1.9 mm; 8 whorls. B.) *Pupa dolium minima* f. curta. "Syntype"
NHMW 27044/2 [labelled as "Original"]. Measures: SH 5.1, SW
2.7, AH 2.0, AW 1.7 mm; 7.5 whorls. C.) *Pupa dolium minima*
f. obesa. "Syntype" NHMW 68376/4 [labelled as "Original"].
Measures: SH 5.6, SW 2.8, AH 2.1, AW 1.8 mm; 8 whorls.

Fig. D-E.   *Orcula dolium kimakoviczi* Brancsik 1887 (assumed synonym of *O.
d. brancsikii*).
[SK], Comitate Trencsin [Trenčín], Manin [Považska Bystrica,
Maninská tiesňava]. D.) *Orcula dolium kimakoviczi* f. curta.
"Syntype" NHMW 68412 (5510) / 27045/8 [labelled as
"Original"]. Measures: SH 7.1, SW 3.1, AH 2.2, AW 2.0 mm; 10.25
whorls. E.) *Orcula dolium kimakoviczi* f. elongata. **"Syntype"**
NHMW 68410 (5440) / 27045/6 [labelled as "Original"]. Measures:
SH 8.3, SW 3.1, AH 2.5, AW 2.1 mm; 10.25 whorls.

Fig. F.   *Orcula dolium brancsikii* Clessin 1887.
[SK], Oberungarn, Comitate Trencsin [Trenčsin], bei Manin
[Považska Bystrica, Maninská tiesňava]. **Syntype ?** NHMW J. N.
22075 (voucher specimen of Clessin's "Molluskenfauna Österreich
Ungarns und der Schweiz", Clessin don. 1894). Measures: SH 7.5,
SW 2.9, AH 2.3, AW 2.0 mm; 10 whorls.

Fig. G-H.   *Orcula dolium titan* (Brancsik 1890).
[SK, Žilinský kraj], Trencsin [Trenčsin], Trencsén-Teplicz
[Trenčianske Teplice], [Mt.] Klepács [Maly Klepač]. Specimens
J-L are all from same locality. G.) *Pupa dolium titan* f. cylindrica.
"Syntype" NHMW 68377 (5448)/3 [labelled as "Original"].
Measures: SH 7.8, SW 4.0, AH 2.9, AW 2.3 mm; 8.5 whorls. H.)
Pupa dolium titan f. obesa. **"Syntype"** NHMW 68413 (5449)/3
[labelled as "Original"]. Measures: SH 8.0, SW 4.2, AH 3.2, AW 2.6
mm, 8.5 whorls.

Fig. I.   *Orcula dolium obesa* (Westerlund 1887).
Ungarn [Ungarn/Hungary formerly comprised several countries
of central-eastern Europe or parts of them]. **Syntype** MNHG
Wstld2086. Measures: SH 8.3, SW 4.0, AH 2.9, AW 2.4 mm; 8.75
whorls.

Plate 3

Plate 4
Pictures Natural History Museum of Vienna (J. HARL)

Fig. A-B.   *Orcula conica conica* (ROSSMÄSSLER 1837).
                  [SLO], Kärnten, Krain [Kranjska - formerly part of Austria]. A.)
                  Syntype SMF 5004/1 LT [JC 332]. Measures: SH 5.9, SW 3.1, AH
                  2.0, AW 1.8 mm; 9.5 whorls. B.) **Syntype** SMF 5005/13. Measures:
                  SH 6.1, SW 3.3, AH 2.2, AW 2.0; 8.5 whorls.

Fig. C.   *Orcula conica* minor ZIMMERMANN 1932.
                  [A], Kärnten, Gurnitzerschlucht bei Sattnitz. **"Holotype"** LML
                  ALT/4915/1. Measures: SH 4.7, SW 2.9, AH 1.8, AW 1.7 mm; 8.5
                  whorls.

Fig. D.   *Orcula restituta* (WESTERLUND 1887).
                  [SLO], Krain, Feistritzthal bei Stein [Kranjska, Kamnik, Kamniška
                  Bistrica valley]: Robič. **Syntype** MNHG Wstld2101. Measures: SH
                  6.3, SW 2.7, AH 2.0, AW 1.6 mm; 9 whorls.

Fig. E.   *Orcula spoliata* (ROSSMÄSSLER 1837).
                  I, Alto Adige [Südtirol], Favogna/Fennberg. NHMW 5820/1.
                  Collected by ZIMMERMANN. Measures: SH 6.6, SW 3.0, AH 2.2, AW
                  2.1 mm; 8.5 whorls.

Fig. F-G.   *Orcula zilchi* URBAŃSKI 1960
                  TR, [Bilecik], between Bozüyük and Inegöl, by the "Mezit 7" bridge,
                  580 m. F) Specimen no. 1. Leg. et det. PÁLL-GERGELY, 2007.09.30.
                  Measures: SH 7.4, SW 3.6, AH 2.5, AW 2.1, 8.5 whorls; pl. 4, fig. F.
                  G) Specimen no. 2. Leg. et det. PÁLL-GERGELY, 2006.05.17. TR, Vil.
                  Bolu, Abant Gölü N., 1030 m. Measures: SH 6.5, SW 3.5, AH 2.3,
                  AW 2.0; 8 whorls; pl. 4, fig. G.

Fig. H.   *Orcula schmidtii schmidtii* (KÜSTER 1843 ?).
                  Montenegro [MNE], Cetinje. NHMW 68433/3. Coll. OBERWIMMER,
                  det. STURANY. Measures: SH 5.2, SW 3.0, AH 1.8, AW 1.9 mm; 8.5
                  whorls.

Fig. I.   *Orcula schmidtii transversalis* (WESTERLUND 1894).
                  [GR], Griechenland, [Pindos mountains], Epirus, road Papingo to
                  [Mt.] Timpfi, Geröllhalde [rocky slope]. NHMW 102.707/5. Leg.
                  SATTMANN, 1988. Det. REISCHÜTZ. Measures: SH 6.1, SW 3.4, AH 2.2,
                  AW 2.0; 8.75 whorls.

Fig. J.   *Orcula jetschini* (KIMAKOWICZ 1883).
                  RO, Transilvania, Cluj-Napoca. **Syntype ?** [according to the label]
                  NHMW Orc. 149/1. Coll. RUSNOV, ex coll. Dr. W. BLUME. Measures:
                  SH 6.1, SW 3.7, AH 2.2, AW 1.9 mm; 8.25 whorls.

Plate 4

Plate 5
Pictures Natural History Museum of Vienna (J. Harl)

Fig. A-G, J *Orcula wagneri wagneri* Sturany in Sturany & Wagner 1914.
A.) Albanien [AL], Mirdita [Mirditë], Berg [Mt.] Munela bei Oroshi [Orosh]; **Holotype** NHMW 41175/1. Leg. Buljabašić, 1905; Measures: SH 6.7, SW 3.3, AH 2.3, AW 2.0 mm; 9 whorls.
B.) Albanien [AL], Mirdita [Mirditë], Zebia [Mali i Zebës] bei Oroshi [Orosh]; **Paratype** NHMW 41186/2. Leg. Buljabašić, 1905; Measures: SH 6.3, SW 3.3, AH 2.4, AW 2.2 mm; 7.75 whorls.
C.) Albanien [AL], Mirdita [Mirditë], Berg Munela [Mt. Munela] bei Oroshi [Orosh]; **Paratype** NHMW 43916/1. Leg. Winneguth,1906; Measures: SH 5.5, SW 3.3, AH 2.1, AW 2.1 mm; 7.25 whorls.
D.) [AL, Kukës], Ljuma-Gebirge [Ljuma mountains], am Drin [besides the Drin river], unterhalb des Koritnik-Gebirges [below the Koritnik mountains]; **Paratype** NHMW 41234/1. Leg. Buljabašić; Measures: SH 5.5, SW 3.0, AH 2.1, AW 2.0 mm; 7.25 whorls.
E.) [AL, Mirditë], N. Albanien, Malšent bei Oroshi [Orosh, Mal i Shêit]; **Paratype** NHMW 41235/1 Leg. Buljabašić, 1904; Measures: SH 4.4, SW 2.8, AH 1.7, AW 1.7 mm; 8 whorls.
F.) [AL, Kukës], N. Albanien, Ljumagebiet [Ljuma mountains], Koritnik-Gebirge [Koritnik mountains] (ca. 2000 m); **Paratype** NHMW 41236/5. Leg. Buljabašić, 1904; Measures: SH 5.5, SW 3.0, AH 2.1, AW 1.9 mm; 7.25 whorls.
G.) Albanien [AL], Mirdita [Mirditë], Berg Zebja [Mali i Zebës] bei Oroshi [Orosh]; **Paratype** NHMW 43917/3. Leg. Winneguth, 1906; Measures: SH 5.7, SW 2.8, AH 2.1, AW 1.8 mm; 8.25 whorls.
J.) [AL], Alban. montenegr. Grenze [Albanian-Kosovarien border], Baštrik [Pashtrik], circa 2700m; **Paratype ?** NHMW 68431/9 [labelled as "original" but not mentioned in the publication]. Leg. Buljabašić, 1904. Measures: SH 6.7, SW 3.1, AH 2.4, AW 2.0 mm; 7.75 whorls.

Fig. H-I.   *Orcula wagneri ljubetenensis* Sturany in Sturany & Wagner 1914. [MK, Macedonian-Kosovarian border], Schar Dagh [Šar planina], Ljubeten [Mt. Ljuboten], nordwestlich von Üsküb [north-west of Skopje].
H.) **Syntype** NHMW 44422/9. Leg. Apfelbeck und Attems, 1906; Measures: SH 6.0, SW 3.0, AH 2.3, AW 2.1 mm; 7.5 whorls.
I.) **Syntype ?** NHMW 68432/6 [labelled as "Original" but not mentioned in the publication]. Leg. Apfelbeck & Attems. Coll. Oberwimmer. Measures: SH 6.1, SW 3.0, AH 2.1, AW 1.8; 8 whorls.

Plate 5

Harl J, Páll-Gergely B, Kirchner S, Sattmann H, Duda M, Kruckenhauser L, Haring E (2014) Phylogeography of the land snail genus *Orcula* (Orculidae, Stylommatophora) with emphasis on the Eastern Alpine taxa: speciation, hybridization and morphological variation. *BMC Evolutionary Biology*, **14**, 223.

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Phylogeography of the land snail genus *Orcula* (Orculidae, Stylommatophora) with emphasis on the Eastern Alpine taxa: speciation, hybridization and morphological variation

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Abstract

Background: The Central and Southern European mountain ranges represent important biodiversity hotspots and show high levels of endemism. In the land snail genus *Orcula* Held, 1837 nine species are distributed in the Alps and a few taxa inhabit the Carpathians, the Dinarids and the Western Black Sea region. In order to elucidate the general patterns of temporal and geographic diversification, mitochondrial and nuclear markers were analyzed in all 13 *Orcula* species. We particularly aimed to clarify whether the Alpine taxa represent a monophyletic group and if the local species diversity is rather the result of isolation in geographically separated Pleistocene glacial refuges or earlier Tertiary and Quaternary palaeogeographic events. In order to test if patterns of molecular genetic and morphological differentiation were congruent and/or if hybridization had occurred, shell morphometric investigations were performed on the *Orcula* species endemic to the Alps.

Results: The phylogenetic trees resulting from the analyses of both the mitochondrial (*COI*, *12S* and *16S*) and the nuclear (*H4/H3*) data sets revealed three main groups, which correspond to the three subgenera *Orcula*, *Illyriobanatica* and *Hausdorfia*. The reconstruction of the historic geographic ranges suggested that the genus originated in the Dinarides during the Middle Miocene and first colonized the Alps during the Late Miocene, giving rise to the most diverse subgenus *Orcula*. Within the latter subgenus (including all Alpine endemics) almost all species were differentiated by both molecular genetic markers and by shell morphometrics, except *O. gularis* and *O. pseudodolium*.

Conclusions: The present study confirms the importance of the Alps as biodiversity hotspot and origin center of land snail diversity. The species diversity in the subgenus *Orcula* was likely promoted by Miocene to Pliocene palaeogeographic events and the insular distribution of preferred limestone areas. In some cases, speciation events could be linked to the divergence of populations in glacial refuges during the Pleistocene. Sporadic contact between geographically separated and reproductively not yet isolated populations led to intermixture of haplogroups within species and even hybridization and mitochondrial capture between species.

Keywords: Integrative taxonomy, Biogeography, Speciation, Hybridization, Morphometric landmark analysis, Glacial refuges, Gastropoda

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Background

European mountain ranges harbor a large number of endemic species and are generally considered as important biodiversity hotspots. Concerning diversity in European terrestrial snails, the IUCN Red List of Threatened Species lists 554 native species from rocky areas, 445 species from shrublands and 367 from forest habitats. Among the first category, rocky areas, the Dinarides represent the most diverse European mountain region with about two hundred native gastropods listed, followed by the Alps and the Carpathians, both with somewhat less than a hundred native species listed [1]. Obvious reasons favoring the diversity in mountain areas are the strong structuring of habitats with a wide range of ecological niches, and the availability of different geological substrates. As most land snails are calciphilous, mountain regions offering limestone bedrock are particularly rich in species and show high rates of endemism, whereas intermediate areas with siliciclastic bedrock constitute migration barriers for many taxa. The isolation in favorable habitats is therefore an important reason for diversification of Alpine land snails [2]. Moreover, the current distribution and diversity patterns of Central European land snails and other biota were affected most strongly by climatic events during the Pleistocene. The shifts in temperature and humidity, and the expansion of glaciers, resulted in the fragmentation of populations of many taxa, complete or local extinction, and the loss of genetic variation due to bottlenecks [3]. As large parts of the Alps were covered by glaciers during the Last Glacial Maximum (LGM; 30–18 kya [4]) and earlier glacial periods, their role as origin center of biodiversity was much discussed in the past decades. Reviews of early molecular genetic studies suggest that the Central European mountain ranges did not provide refuges during glacial maxima, but were settled recently from more southern regions [3,5,6]. However, more recent molecular genetic studies support the presence of northern refuges in the periphery of the Alps and in the Western Carpathians for particular organisms like plants and invertebrates [7-12]. Populations of former refuge areas were usually characterized by high genetic diversity and the presence of rare (private) alleles [13].

In the present study we investigate the phylogeny and phylogeography of the calciphilous land snail genus *Orcula* Held, 1837. *Orcula* species are high-spired snails, 5 to 10 mm in height, with internal lamellae extending to the aperture margins. The morphologies of these lamellae serve as the primary characters for species identification. Currently, 13 species are known, almost all distributed either in the Alps, the Carpathians or in the Dinarides. Only a single species, *Orcula zilchi* Urbański, 1960, was recorded from the western Black Sea region of Bulgaria and Western Anatolia. *Sphyradium dobrogicum* Grossu, 1986 was also

classified within the genus *Orcula* in the Fauna Europaea checklist [14], but without any published reference. However, based on available information, the species was synonymized with *Sphyradium doliolum* (Bruguière, 1792) [15]. Data on type specimens and taxonomic considerations about all *Orcula* taxa are summarized in the type catalogue of [16].

So far, the most comprehensive investigation of the genus was performed by Gittenberger [17], who attempted to differentiate the Alpine *Orcula* taxa and the Dinaric *Orcula schmidtii* (Küster, 1843) by anatomical and shell morphological traits. Schileyko [18] also investigated the genital anatomy and formulated hypotheses about the relatedness of several species. Páll-Gergely et al. [15] were the first to study the anatomy of *Orcula jetschini* (Kimakowicz, 1883) and *O. zilchi* and, based on differences in the morphology of the penial caecum, the shell structure and the morphology of the aperture folds, subdivided the genus into three subgenera: (1) *Orcula*, (2) *Illyriobanatica* Páll-Gergely & Deli 2013 and (3) *Hausdorfia* Páll-Gergely & Irikov 2013. The subgenus *Orcula* includes all species distributed in the Alps, among them the type species *Orcula dolium* (Draparnaud, 1801), which has by far the widest distribution including the Alps, the Western Carpathians and surrounding lowlands [19]. In contrast, the Alpine endemics are almost exclusively restricted to rocky limestone habitats of the Northern and the Southern Calcareous Alps. Their distribution was mainly investigated by Zimmermann [20] and Klemm [2]. *Orcula gularis* (Rossmässler, 1837) shows a disjunct distribution in both the Northern Calcareous Alps (Salzburg, Styria and Upper Austria) and the Southern Calcareous Alps (Carinthia and East Tyrol). *Orcula austriaca* Zimmermann, 1932 shows a similar distribution but its main area is situated more easterly in the Northern Calcareous Alps of Lower Austria. *Orcula pseudodolium* Wagner, 1912 inhabits a few mountains in the Northern Calcareous Alps of Upper Austria only. A fourth species, *Orcula fuchsi* Zimmermann, 1931, is restricted to two mountains (Mt. Gippel and Mt. Göller) in the Northern Calcareous Alps of Lower Austria. The other four Alpine endemics are exclusively found in the Southern Calcareous Alps of Austria, Slovenia and Italy. Of these, *Orcula tolminensis* Wagner, 1912 stands out as it resembles conchologically a dwarf form of *O. gularis*, with similar aperture characteristics; it is known from three sites in Southern Carinthia and Slovenia only. *Orcula restituta* (Westerlund, 1887) is mainly distributed in the Slovenian Alps and *Orcula spoliata* (Rossmässler, 1837) has an isolated distribution about 200 km west in Trentino-Alto Adige (Italy). The fourth *Orcula* species of the Southern Calcareous Alps is *Orcula conica* (Draparnaud, 1801). It is common in the eastern part of the Southern Calcareous Alps, but was found at a single site in the Dinarides around the Plitvice lakes (Republic of Croatia) as well. The subgenus

Illyriobanatica comprises *O. schmidtii, Orcula wagneri* Sturany, 1914 and *O. jetschini. O. schmidtii* and *O. wagneri* inhabit high mountain regions of the Dinarides, from the Republic of Serbia to southern Greece, and their distribution ranges do greatly overlap [21]. A delimitation of the two taxa is problematic, because the shell characters are highly variable and do not allow to clearly distinguish the two species (see pictures in [16] and [21]). Therefore, the latter two taxa are referred to as *O. wagneri/schmidtii* complex in the following. *O. jetschini* was reported from Romania and is the only *Orcula* species of the Western Romanian Carpathians. It is distributed in the Banat region, western Transylvania (including Crisana) and northern Oltenia. Its shell shows similarities to those of the Dinarid species *O. wagneri* and *O. schmidtii*, but it is a woodland species of lower elevations, occurring mainly among leaf-litter or decaying dead wood. *Orcula zilchi* represents the monotypic subgenus *Hausdorfia*. It is known from three localities in South-Eastern Bulgaria and from three sites in Western Anatolia only. Its habitat ranges from leaf litter of alluvial forests in the western Black Sea region to limestone rocks in Western Turkey [15,22].

The present paper addresses the evolutionary history of the genus *Orcula* in general as well as the phylogeographic patterns of the species endemic to the Alps in particular. We aimed to answer the following questions: Which geographic areas were inhabited by ancestral populations of *Orcula*? What are the causes for the high species diversity in the Alpine region? Is there a congruency between molecular genetic patterns and morphologically defined groups in the Alpine *Orcula* species? Are there indications for recent or past hybridizations between any of the species?

We performed the first phylogeographic study of the genus *Orcula* based on comprehensive mitochondrial (mt) and nuclear (nc) data sets including material of all 13 species. A molecular clock analysis was performed and combined with a phylogeographic range reconstruction to trace the distribution patterns of the mt lineages throughout time.

In order to test whether the differentiation in shell morphology is congruent with the molecular genetic groupings, morphometric landmark analyses were conducted with the group of *Orcula* species endemic to the Alps and the Alpine-Dinarid *O. conica*.

Results

Phylogenetic trees

A 655 bp fragment of the mitochondrial (mt) cytochrome c oxidase subunit I (*COI*) was analyzed in 295 specimens from 151 sites (Figure 1 and Table 1), including samples of all 13 extant *Orcula* species (Figure 2). The sequences of the *Orcula* species endemic to the Alps and the Alpine-
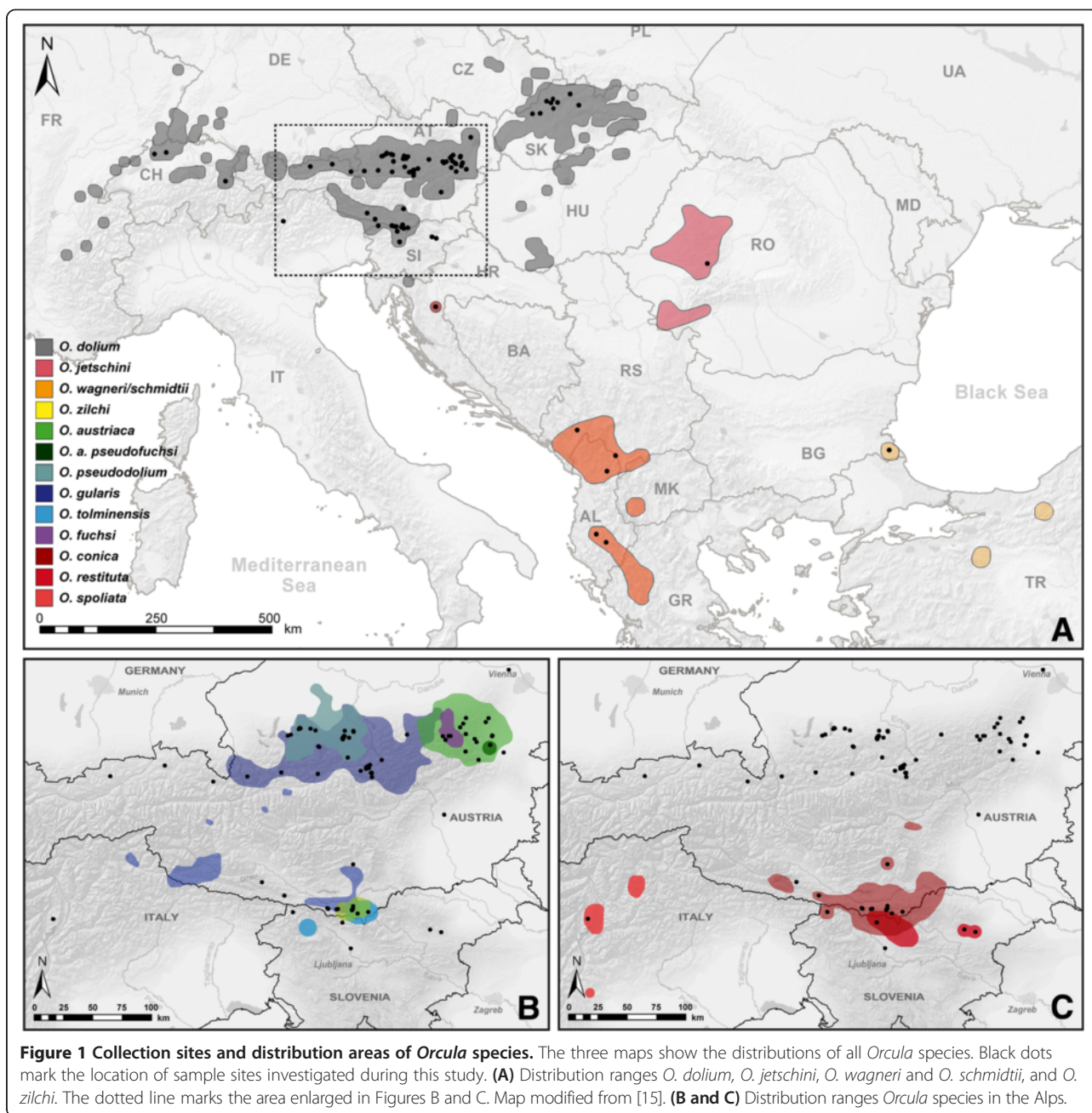
Dinarid *O. conica* constituted three quarters of the samples. The nine species were in the focus of the study and we aimed to infer the degree of intraspecific molecular genetic (mtDNA) and morphological variation across the major parts of their distribution areas.

In the Bayesian Inference (BI) and Maximum Likelihood (ML) trees calculated with the *COI* data set only, most species clades are well supported (data not shown), but the relationships between the clades are not resolved. The complete *COI* data of the seven Alpine endemics and *O. conica* are shown separately as phylogenetic networks.

In order to set up a reliable phylogenetic framework, we additionally analyzed sections of the mt *12S* ribosomal RNA (*12S*), *16S* ribosomal RNA (*16S*) and the nuclear (nc) histone *H3* and *H4* complex (*H4/H3*) in a selection of 87 individuals. We selected a representative sample of the major mt *COI* lineages and geographic distributions (including most type localities). Substitution saturation in the mt *COI* and the trimmed *12S* and *16S* alignments was examined with the test of Xia et al. [23], implemented in MEGA v.5.1 [24]. The alignments show only little substitution saturation, with $I_{ss.c}$ values significantly larger than I_{ss} values ($P = 0.000$): *12S* ($I_{ss.c}$ 0.699 > I_{ss} 0.218), *16S* ($I_{ss.c}$ 0.722 > I_{ss} 0.249) and *COI* ($I_{ss.c}$ 0.718 > I_{ss} 0.271). However, moderate substitution saturation ($P = 0.0012$) is observed in the 3rd codon positions of the *COI* with $I_{ss.c}$ (0.686) and I_{ss} (0.581) values differing only marginally between each other.

The BI and ML phylograms calculated with the concatenated alignments (*COI*, *12S* and *16S*) show congruent, well resolved topologies with high support values for most of the nodes (Figure 3). The topology remains the same when the 3rd codon position of the *COI* is excluded from the data set, but most nodes show lower posterior probabilities or likelihood values, respectively (Additional file 1). The nc trees were calculated with the *H4/H3* data of the same subset of specimens. The overall topology with a division into three main clades representing the three subgenera *Orcula*, *Illyriobanatica* and *Hausdorfia* is illustrated by the nc tree as well (Figure 4). However, the nc data set is less variable, and support values for most nodes are lower in the nc tree:

The first main clade in the mt and nc trees corresponds to the subgenus *Orcula*, which comprises all *Orcula* species showing distributions in the Alps. *O. dolium* is clearly monophyletic and the sister group to a clade comprising the lineages of the Alpine endemics and the Alpine-Dinarid species *O. conica*. In the mt trees (Figure 3, Additional file 1), *O. conica* branches off from the basal node, whereas in the nc tree *O. spoliata* and *O. restituta*, the two being sister species, split basally (Figure 4). In the mt trees, the most basal nodes are weakly supported, while the relationships between the other Alpine endemics are well resolved: *O. fuchsi*, endemic to the Northern

Figure 1 Collection sites and distribution areas of *Orcula* species. The three maps show the distributions of all *Orcula* species. Black dots mark the location of sample sites investigated during this study. **(A)** Distribution ranges *O. dolium, O. jetschini, O. wagneri* and *O. schmidtii*, and *O. zilchi*. The dotted line marks the area enlarged in Figures B and C. Map modified from [15]. **(B and C)** Distribution ranges *Orcula* species in the Alps.

Calcareous Alps, is the sister group of a highly supported clade comprising *O. austriaca, O. gularis, O. pseudodolium* and *O. tolminensis*. Within this clade, *O. gularis* is paraphyletic because most specimens of *O. gularis* and *O. pseudodolium* show up in the same clade. Moreover, three specimens of *O. gularis* (IndIDs 377, 885 and 924) from three distinct sites (ENN4, ENN21 and ENN23; Styria, Austria) possess different mt haplotypes, being closely related to those of *O. tolminensis. O. austriaca* forms the sister clade of the latter two clades. Differing in a few substitutions or indels only, the *H4/H3* sequences do not allow resolving the relationships within

the group of the latter species (Figure 4). However, this part of the *H4/H3* tree shows some peculiar patterns. There is a rather deep split between samples of *O. austriaca* from the Northern and the Southern Calcareous Alps, whereas these geographically isolated populations are barely differentiated in the mt sequences. There is also a larger diversity of nc haplotypes within *O. pseudodolium* when compared to the more widespread *O. gularis*.

The second main clade corresponds to the subgenus *Illyriobanatica* and includes *O. schmidtii* and *O. wagneri* from the Dinarides, and *O. jetschini* from the Western Romanian Carpathians. The sequences of *O. schmidtii*

Table 1 Sampling localities and individuals

Locality code	Locality	WGS84 (N)	WGS84 (E)	m asl	IndID
Western Romanian Carpathians					
BAN1	RO, Bihor, Şuncuiuş, valley of Crisul Repede	45°56.405′	22°32.954′	200	**6646**, **6647** j
Strandzha Mountains					
STZ1	BG, Burgas, Strandzha Mts, Kondolovo vill.	42°6.144′	27°39.888′	330	**7021**, **7022**, **7023** z
Dinarides					
GJA1	AL, Kukës, Bicaj, Gjalica e Lumës	41°59.959′	20°27.680′	2060	**1393**, **1394** sch
MLK1	HR, Lika Senj, Plitvička Jezera NP, Galovac lake	44°52.616′	15°36.389′	573	3336, 3337, **3338** c
PRO1	ME, Podgorica, Rikavačko, summit area	42°34.483′	19°35.044′	1800	**3543**, **3544** w
PTK1	KO, Prizren, Paštrik, summit	42°13.204′	20°31.572′	1986	**3538** w
TOM1	AL, Berat, Tomor Mts, Tomor summit area	40°42.548′	20°8.662′	2200	**3541**, **3542** w
TOM2	AL, Berat, Tomor Mts, Maja e Ramiës	40°34.100′	20°15.522′	1850	**7138**, **7139** sch
Northern Calcareous Alps					
MRZ1	AT, NOE, Göller W-side, Gscheid	47°48.622′	15°27.084′	914	3024, 3025, 3026 a
MRZ2	AT, NOE, Göller S-side	47°46.941′	15°28.833′	1365	2955, **2956** a; 2951, **2952**, 2953 f
MRZ3	AT, NOE, Göller N-side, Turmmauer	47°48.705′	15°31.118′	812	3029, 3032, 3034 a; 3033, **3036**, 3037, 3038 f
MRZ4	AT, NOE, Göller N-side, Klopfermauer	47°48.705′	15°32.264′	741	2957, **2958**, 2959 a
MRZ5	AT, NOE, Göller N-side, Klopfermauer waterfall	47°48.705′	15°32.264′	741	2965, 2966, 2967, 2968 f
BGD4	AT, S, Saalfelden, Einsiedler	47°26.622′	12°51.720′	1029	**4119** d
DAC3	AT, OOE, Wiesberghaus, Wiesbergalm	47°31.529′	13°37.493′	1685	**1279** d
DAC4	AT, ST, Grimming, Grimming SE-side	47°31.172′	14°2.326′	1149	**5643** d; 5644, 5645 g
ENN3	AT, ST, Johnsbachtal, Langries-Brücke	47°33.681′	14°34.845′	631	5649, 5650, 5651 g
ENN4	AT, ST, Johnsbachtal, Kaderalpl	47°34.063′	14°34.899′	634	884, 886 g; **885** g*
ENN8	AT, ST, Johnsbachtal, Hellichter Stein	47°34.544′	14°35.348′	606	907, 908, 909 g
ENN9	AT, ST, Johnsbachtal, Gseng	47°34.085′	14°35.687′	766	5619, 5620, 5621 g
ENN10	AT, ST, Johnsbachtal, Upper Gseng	47°34.033′	14°36.106′	1039	5629, 5630, 5631 g
ENN11	AT, ST, Haindlkar, Zigeuner	47°35.114′	14°36.695′	603	898
ENN12	AT, ST, Ennstal, Haindlkar-Hütte 2	47°34.039′	14°36.773′	1078	5635, 5636, 5637 g
ENN20	AT, ST, Admonter Kalbling, Kalbling	47°32.678′	14°31.276′	1746	1354, 1355, 1356 g
ENN21	AT, ST, Johnsbachtal, Langries-bridge	47°33.681′	14°34.845′	667	375, **376** g; **377** g*
ENN22	AT, ST, Johnsbachtal, Langgrieß-estuary	47°33.666′	14°34.920′	652	631, 1937 g
ENN23	AT, ST, Johnsbachtal, Im Gseng 1	47°34.044′	14°34.934′	634	**924** g*
ENN24	AT, ST, Johnsbachtal, Im Gseng 2	47°34.266′	14°35.098′	613	899, 901 g
ENN25	AT, ST, Gstatterboden, Rauchbodenweg	47°35.493′	14°37.594′	587	612, 1130, 1353 g
ENN26	AT, ST, Johnsbachtal, Schneckengraben	47°31.782′	14°38.258′	1035	3090, 3091, 3092 g
ENN27	AT, ST, Johnsbachtal, Teufelsschlucht	47°31.648′	14°38.615′	969	3098, 3099, 3100 g
ENN28	AT, ST, Johnsbachtal, Teufelsklamm	47°31.843′	14°38.697′	1028	329, 330, **331** g
ENN29	AT, ST, Johnsbachtal, Ebner Mäuer	47°31.657′	14°38.818′	1061	3095, 3097 g
ENN30	AT, ST, Leobner, Leobner Mauer	47°29.861′	14°38.959′	2009	1350, 1351, **1352** g
ENN31	AT, ST, Hieflau, Wandaubruecke	47°37.715′	14°45.468′	488	3102, 3103, **3104** g
FOB1	AT, ST, Schöckl, Teufelstein	47°12.525′	15°27.665′	950	**858** d
FOB3	AT, NOE, Sonnwendstein, Almweg 1229	47°37.604′	15°51.141′	1229	**370** d
GUT1	AT, NOE, Tiefental, Ochbauer	47°52.638′	15°38.854′	739	**3042** d
GUT2	AT, NOE, Halbachtal, Rossbachklamm 1	47°54.327′	15°40.937′	649	**3070**, 3071, 3072 a

Table 1 Sampling localities and individuals *(Continued)*

GUT3	AT, NOE, Halbachtal, Kleinzell	47°56.902′	15°42.874′	507	3044, 3045 a
GUT5	AT, NOE, Gösing, foot of the hill, W-side	47°44.431′	15°59.198′	700	**5476**, 5479 ap
GUT6	AT, NOE, Gösing, W-side	47°44.401′	15°59.205′	864	2938, **2939**, 2940, 2941 ap
GUT8	AT, NOE, Grafenberg, Seiser Toni	47°48.360′	16°0.503′	780	1115 a
GUT9	AT, NOE, Große Kanzel, Springlessteig 1	47°48.800′	16°0.600′	720	638, 639, 832, 2812, 2813 a
GUT10	AT, NOE, Große Kanzel, Johann-Stich ladder	47°48.649′	16°0.781′	820	598 a
GUT11	AT, NOE, Falkenstein, Herrengrotte	47°49.528′	15°42.674′	749	3065 a
GUT12	AT, NOE, Pernitz, Hirschwände	47°54.955′	15°56.297′	640	5660 a
KAI4	AT, T, Wilder Kaiser, Hochgrubach	47°33.357′	12°18.604′	1666	**1981** d
KWL1	AT, T, Pertisau, Achensee W-shore	47°27.423′	11°42.081′	955	**615** d
LEC1	AT, T, Imst, Hahntennjoch	47°17.225′	10°36.566′	1482	**5932** d
OOV1	AT, OOE, Traunstein, Naturfreunde-Steig	47°51.581′	13°49.056′	631	340, 341, 342 g
OOV3	AT, OOE, Traunstein, Mairalmsteig	47°51.960′	13°50.047′	1166	633, 634, 635 g
OOV4	AT, OOE, Traunstein, Naturfreunde-Steig (arch)	47°52.135′	13°49.591′	1482	359, 618 g
OOV5	AT, OOE, Hoher Nock, Feichtausee	47°47.550′	14°18.716′	1399	3360, 3361, 3362 p
OOV6	AT, OOE, Hoher Nock, Hauptkar 2	47°47.307′	14°18.953′	1733	3368, 3370, 3371 p
OOV7	AT, OOE, Hoher Nock, Feichtau (Seeweg)	47°47.783′	14°19.044′	1418	3364, 3365, 3366 p
OOV8	AT, OOE, Hoher Nock, Schneeberg	47°47.173′	14°19.048′	1877	3449, 3450, 3451 p
OOV9	AT, OOE, Hoher Nock, Feichtausee-Hütte 1	47°48.062′	14°19.144′	1360	2806, 2807, 2808 p
OOV10	AT, OOE, Hoher Nock, Feichtausee-Hütte 2	47°48.062′	14°19.144′	1360	3356, 3357, 3358, **3359** p
OOV11	AT, OOE, Hoher Nock, Haltersitz	47°47.466′	14°19.204′	1583	3352, 3353, 3354 p
OOV12	AT, OOE, Hoher Nock, Haltersitz 1	47°47.866′	14°19.260′	1388	3348, 3349, 3350 p
OOV13	AT, OOE, Hoher Nock, Eiseneck 1	47°48.755′	14°19.757′	1152	**3340**, 3341, 3342 p
OOV14	AT, OOE, Hoher Nock, Eiseneck 2	47°48.629′	14°20.182′	1298	**3344**, 3345, 3346 p
OOV15	AT, OOE, Krumme Steyrling-Tal, Kienberg	47°51.042′	14°20.308′	486	3453, 3454, 3455 p
OOV16	AT, OOE, Bodinggraben, Scheiblingau	47°47.883′	14°23.217′	580	5858, 5862, 5869, **5871** p
OOV17	AT, OOE, Stefflkogel, Grosser Weissenbach 2	47°50.967′	14°26.050′	440	**5827**, 5829, 5830 p
OOV18	AT, OOE, Stefflkogel, Grosser Weissenbach 1	47°51.050′	14°26.217′	420	**5819**, 5820, 5821 p
OOV19	AT, OOE, Stefflkogel, Unterer Zöbelgraben	47°50.750′	14°26.300′	550	**5838**, 5841 p
OOV20	AT, OOE, Stefflkogel, Oberer Zöbelgraben	47°50.633′	14°26.317′	720	5852, **5855** p
OSR3	AT, NOE, Seebenstein, Türkensturz	47°40.876′	16°8.277′	550	**5606** d
SNH21	AT, NOE, Fadensteig, Fadenwände 1525	47°47.253′	15°48.655′	1525	1359, 1361 a; **1362** d
SNH24	AT, NOE, Rax, Bismarcksteig	47°41.537′	15°42.426′	1787	323, **324**, 325, 327, 328, 830 a
SNH26	AT, NOE, Schneeberg, Hahnriegel	47°45.799′	15°49.928′	1804	6154, 6155, 6156 a
SZK8	AT, OOE, Hochschneid, Hochschneid	47°48.458′	13°41.533′	1624	**347** d
SZK10	AT, OOE, Feuerkogel, Pledialm	47°48.984′	13°43.549′	1444	1147 g
TEN2	AT, S, Hochthron, Thronleiter	47°29.310′	13°14.611′	1940	**1930** g
TOT1	AT, ST, Großer Priel, Vorderer Ackergraben	47°43.724′	14°2.504′	1027	3867, 3868, 3869 g
TOT3	AT, ST, Großer Priel, Welser-Hütte	47°43.493′	14°2.990′	1747	**3873** d; 3875, 3876, 3877 g
TOT4	AT, OOE, Grünau, Grünau S	47°51.867′	13°56.906′	530	6153 g
TOT5	AT, OOE, Grünau, Kasberg-Almtal	47°51.076′	14°0.365′	600	6151, **6152** g
WIW2	AT, NOE, St. Andrä-Wördern, Hagenbachklamm	48°18.660′	16°12.582′	191	**392** d
WIW4	AT, NOE, Steinwandgraben, Teufelsbrücke	47°56.711′	15°58.341′	672	5661, 5662 a
YBB7	AT, NOE, Lechnergraben, Talschluss	47°49.387′	15°2.673′	1160	89, 90, 91, 1113, 1114 g

Table 1 Sampling localities and individuals *(Continued)*

Southern Calcareous Alps					
GAl1	AT, K, Kreuzen, Meierle	46°41.028′	13°26.015′	927	**1131** d
GAl4	AT, K, Dobratsch, Höhenrain	46°35.048′	13°41.041′	1900	**640** d
JUL4	SI, Dežela Kranjska, Triglav, Kluže	46°11.186′	14°25.474′	601	**1363** d
JUL5	SI, Dežela Kranjska, Vršič, N-side	46°27.558′	13°46.883′	960	1390, 1391 c
JUL6	SI, Dežela Kranjska, Trišič, Čadovlje	46°23.025′	14°19.776′	624	3473, **3474** r
KWN1	AT, K, Koschuta, Trögener Klamm	46°27.212′	14°29.811′	762	3899, 3900, 3901 c; **3905** d
KWN2	AT, K, Bärental, oberhalb Stausee	46°29.621′	14°9.708′	650	6142, 6143 g
KWN3	AT, K, Unteres Bärental, Bärental	46°29.227′	14°10.270′	770	**1938**, 1939 c
KWN4	AT, KR, Loibltal, Tscheppa gorge (Sapotnitza)	46°29.120′	14°15.810′	695	3469, 3470, **3471** c
KWN5	AT, KR, Loibltal, Tscheppa gorge	46°29.287′	14°16.680′	590	1525, 1526, 1527 c; 1950, 1951, 1952 g
KWN6	AT, ST, Großer Pyhrgas, Lugkogel	47°38.784′	14°22.431′	1424	3086, 3087, 3088 g
KWN7	AT, K, Hochobirmassiv, Freibachgraben 3	46°29.025′	14°26.049′	808	590, 591, **592** af
KWN8	AT, K, Kuhberg, Zell-Freibach	46°28.714′	14°26.092′	1001	3457,3458, 3459 af
KWN9	AT, K, Hochobir, Freibachgraben 2	46°49.877′	14°26.615′	788	4095, 4096, 4097 c
KWN10	AT, K, Hochobir, Freibachgraben 1	46°29.358′	14°26.748′	801	4091, 4092, 4093 af
KWN11	AT, K, Hochobir, Oberer Ebriachbach	46°30.630′	14°26.803′	900	3895, 3896, **3897** af; 3891, **3892**, 3893 t
KWN12	AT, K, Eisenkappel, Kupitzklamm	46°27.979′	14°36.915′	674	**4075**, 4076, 4077 t
STE1	SI, Dežela Kranjska, Solčava, Bela	46°19.906′	15°17.862′	751	1370, **1371**, 1379 r
STE2	SI, Dežela Kranjska, Solčava, Robanov Kot	46°18.682′	15°25.213′	1095	2824, 2825, **2826** r
VDN1	IT, Trentino, Sfruz, Sfruz-Credai	46°20.892′	11°8.387′	1070	5655, **5656**, **5657**, 5658 sp
Western Alps					
GLA1	CH, Sankt Gallen, Calfeisental, St. Martin	46°55.353′	9°21.333′	1347	**5934** d
SML4	CH, Bern, Moutier, Gorges de Court	47°15.360′	7°20.610′	650	**6144, 6145** d
SML11	CH, Bern, Rumisberg, Schore	47°16.671′	7°38.210′	1066	**6140** d
Western Carpathians					
MLF1	SK, TN, Povazská Bystrica, Považský hrad N	49°8.734′	18°27.422′	500	**3938, 3939, 3942** d
MLF2	SK, TN, Povazská Bystrica, Manínska tiesňava S	49°8.398′	18°30.421′	380	**3919** d
MLF3	SK, TN, Povazská Bystrica, Manínska tiesňava N	49°8.366′	18°30.475′	400	**3915** d
MLF4	SK, ZI, Súľov-Hradná, Súľovské skaly	49°10.101′	18°34.633′	315	**3932** d
MLF6	SK, ZI, Rajecké Teplice, Skalky Strážovské S	49°8.115′	18°41.745′	470	**3947** d
MLF7	SK, ZI, Malá Fatra, Terchová-Vrata	49°14.664′	19°2.360′	564	**1372, 1373, 1376** d
STR1	SK, TN, Trenčianske Teplice, Malý Klepáč W	48°53.720′	18°10.649′	480	**3909** d
STR2	SK, TN, Strážovské vrchy, Valaska Beta	48°53.519′	18°22.469′	445	**1996** d
VEF1	SK, ZI, Ružomberok, Cebrat S-side	49°5.474′	19°17.174′	700	**3926** d
OUTGROUP TAXA					
Dinarides					
PIN1	GR, West Makedonia, Florina, Petres	40°44.066′	21°40.699′	580	**7002, 7003, 7004** *Orculella bulgarica*
Northern Calcareous Alps					
SNH23	AT, NOE, Semmering, Adlitzgraben	47°39.361′	15°50.168′	650	**833** *Sphyradium doliolum*
Pontic Mountains (Turkey)					
PON1	TR, Erzurum, Aşkale, Tercan tunnel	39°50.396′	40°33.984′	1880	**6606, 6607** *Orculella bulgarica*
PON2	TR, Erzurum, Aşkale, Aşkale	39°56.472′	40°36.395′	1652	**7105** *Orculella bulgarica*

Table 1 Sampling localities and individuals *(Continued)*

Baetic System (Spain)						
BAE1	ES, Andalusia, Granada, Barrio los Parrales	37°18.437′	−3°14.807′	940	**7132** *Orculella aragonica*
BAE 2	ES, Andalusia, Granada, Barranco de las Ramillas	37°12,471′	−3°21.626′	1431	**7133** *Orculella aragonica*
BAE 3	ES, Andalusia, Granada, Cortijo del Olivar	37°31.952′	−2°44.382′	1431	**7135** *Orculella aragonica*
BAE 4	ES, Andalusia, Granada, La Torre spring	37°15.164′	−3°23,673′	1365	**7137** *Orculella aragonica*

Abbreviations

Mountain range: BAE, Baetic System; BAN, Banat; BGD, Berchtesgaden Alps; DAC, Dachstein Mts.; ENN, Ennstal Alps; FOB, Fischbach Alps; GAI, Gailtal Alps; GJA, Gjallica; GLA, Glarner Alps; GUT, Gutenstein Alps; JUL, Julian Alps; KAI, Kaiser Mts.; KWL, Karwendel; KWN, Karawanks; LEC, Lechtal Alps; MLF, Malá Fatra; MRZ, Mürzsteg Alps; OOV, Upper Austrian Prealps; OSR, Eastern Styrian Alps; PRO, Prokletje Mts.; PTK, Paštrik; SML, Swiss Plateau; SNH, Rax-Schneeberg Alps; STE, Steiner Alps; STR, Strážovské vrchy; STZ, Strandza Mts.; SZK, Salzkammergut Mts.; TEN, Tennengebirge; TOM, Tomor Mts.; TOT, Totes Gebirge; VDN, Valle di Non; VEF, Veľká Fatra; WIW, Wienerwald; YBB, Ybbstal Alps.

Country names: AL, Albania; AT, Austria; BG, Bulgaria; CH, Switzerland; DE, Germany; HR, Croatia; IT, Italy; KO, Kosovo; ME, Montenegro; RO, Romania; SI, Slovenia; SK, Slovakia.

Federal districts: BRN, Bern; BY, Bayern; FVG, Friuli-Venezia Giulia; K, Kärnten; KR, Kranjska; NOE, Niederösterreich; OOE, Oberösterreich; S, Salzburg; SG, St. Gallen; ST, Steiermark; T, Tirol; TN, Trenčiansky kraj; ZI, Žilinský kraj.

List of localities and individuals included in the present study. The first column indicates the geographic region. Each locality is defined by a unique locality code, representing a single collection site in one of the geographic (mountain) regions investigated. The locality column provides information on the respective localities. The GPS coordinates are given according to the World Geodetic System 1984 (WGS84) alongside the altitude in meters above sea level (asl). The individual IDs (IndIDs) of the specimens, together with information on the respective taxon names are provided for each locality. IndIDs in bold letters indicate that the complete marker set (*COI, 12S, 16S, H4/H3*) was sequenced in the respective specimens. Abbreviations of taxon names: a: *austriaca*, af: *austriaca faueri*, ap: *austriaca pseudofuchsi*, c: *conica*, d: *dolium*, f: *fuchsi*, g: *gularis*, g*: *gularis* (clade 2), j: *jetschini*, p: *pseudodolium*, r: *restituta*, sch: *schmidtii*, sp: *spoliata*, t: *tolminensis*, w: *wagneri* and z: *zilchi*.

and *O. wagneri* form a highly supported clade in both the mt and the nc trees, but the two species are not monophyletic. Consequently, they are referred to as *O. schmidtii/ wagneri* complex in the present study. *O. jetschini* is clearly separated from the *O. schmidtii/wagneri* clade, but the monophyly of the subgenus *Illyriobanatica* is well supported (Figures 3, 4). Apart from other patterns of sequence similarity, all taxa of the subgenus *Illyriobanatica* lack a section of approximately 230 bp in the non-coding *spacer* region of the *H4/H3* sequences.

A third major lineage is constituted by *O. zilchi*, representing the monotypic subgenus *Hausdorfia*. It inhabits the western Black Sea region and is not closely related to any other species in the trees. Similarly as the taxa of the subgenus *Illyriobanatica*, *O. zilchi* features a very short branch in the *H4/H3* tree (Figure 4), but not in the mt trees (Figure 3, Additional file 1).

Molecular clock analysis and reconstruction of geographic range history

Molecular clock analyses and reconstructions of the geographic range histories were performed to analyze the temporal and geographic patterns of divergence of the mt lineages. The molecular clock dated trees were calculated
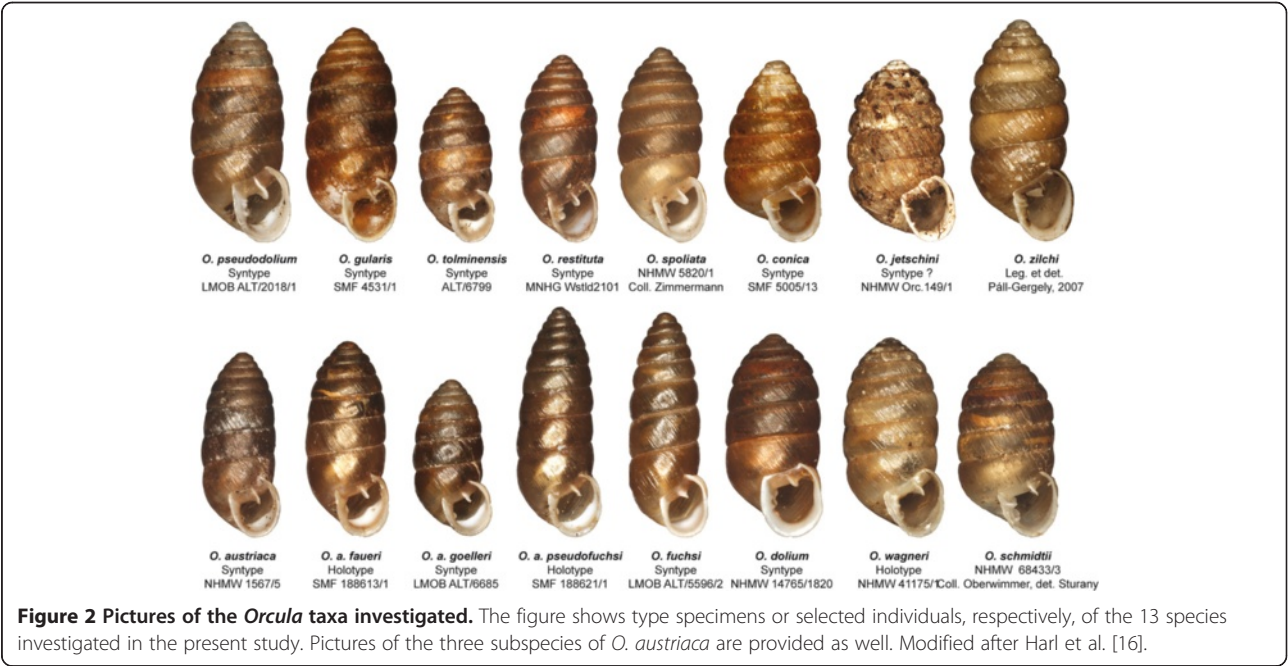

Figure 2 Pictures of the *Orcula* taxa investigated. The figure shows type specimens or selected individuals, respectively, of the 13 species investigated in the present study. Pictures of the three subspecies of *O. austriaca* are provided as well. Modified after Harl et al. [16].
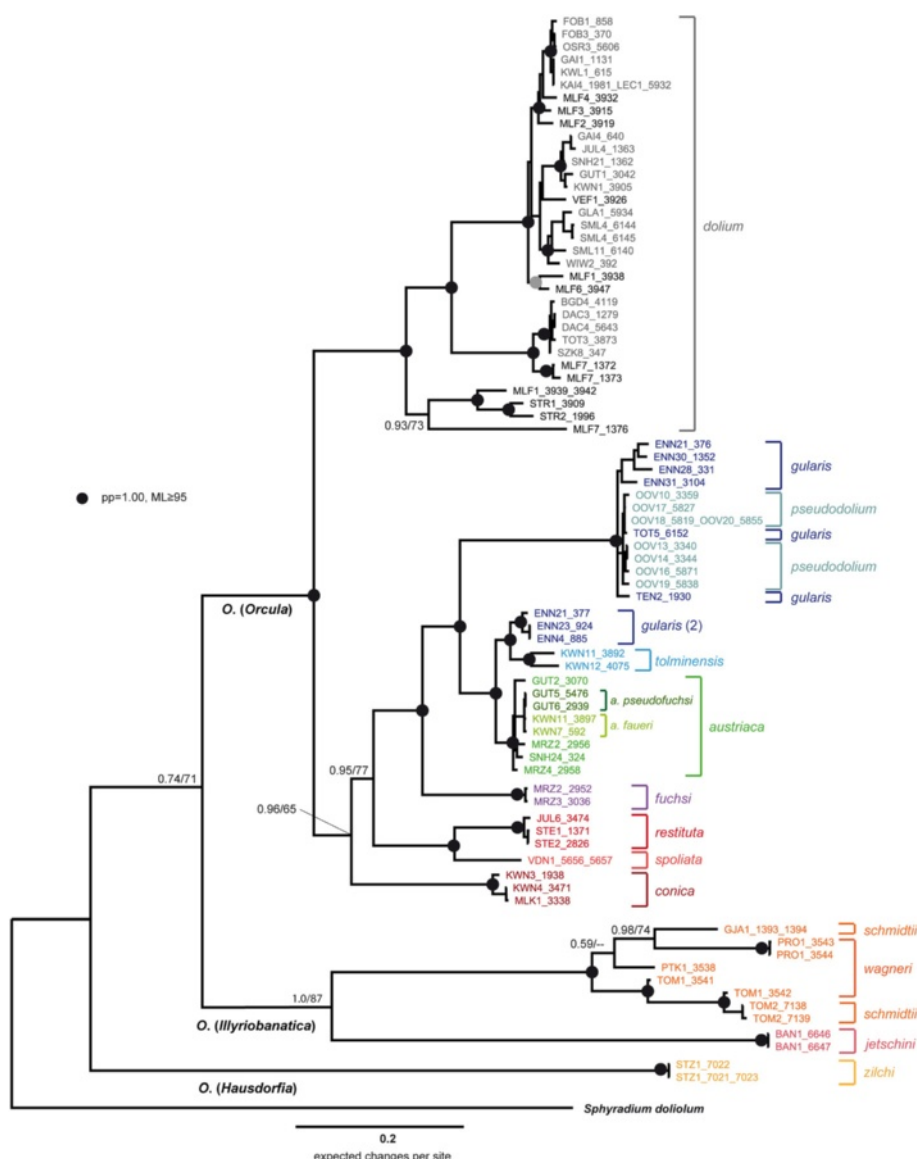
Figure 3 BI tree of the concatenated mitochondrial sequences (*12S*, *16S* and *COI*). Posterior probabilities and ML bootstrap values are provided for all nodes above species level. The scale bar indicates the expected number of substitutions per site according to the models of sequence evolution applied. The black and grey dots indicate nodes with high BI posterior probabilities and ML bootstrap values (see Figure). The colors of the specimen labels correspond to those used in Figures 4, 7 and 9.

in BEAST v1.7.5 [25] with the three mt markers (*COI*, *16S* and *12S*), using *Sphyradium doliolum*, *Orculella bulgarica* (Hesse, 1915) and *Orculella aragonica* (Westerlund, 1897) as outgroups. Since the inclusion of the sequences of the two *Orculella* species as additional outgroups affected the patterns in the *12S* and *16S* alignments, the resulting maximum clade credibility trees differ in their topology from the phylograms: The clade of the subgenus *Illyriobanatica* branches off from the basal node in the molecular clock trees (Figure 5, Additional files 2 and 3), whereas the subgenus *Hausdorfia* takes this position in the mt phylograms (Figure 3, Additional file 1). However, the node marking

the first split within the genus *Orcula* obtained rather low support in all (also the nc histone) trees, and the relation between the three subgenera can still be considered as unresolved.

Three different approaches were performed to estimate the divergence times: (1) In the first approach (Additional file 2), the root of the tree (node XIV) was calibrated to the age of the fossil *Nordsieckula falkneri* (Hausdorf, 1995), the presumed most recent common ancestor of *Orcula*, *Sphyradium* and *Orculella*. The mean age of the node marking the split of *Illyriobanatica* from the subgenera *Orcula* and *Hausdorfia* (node I) was
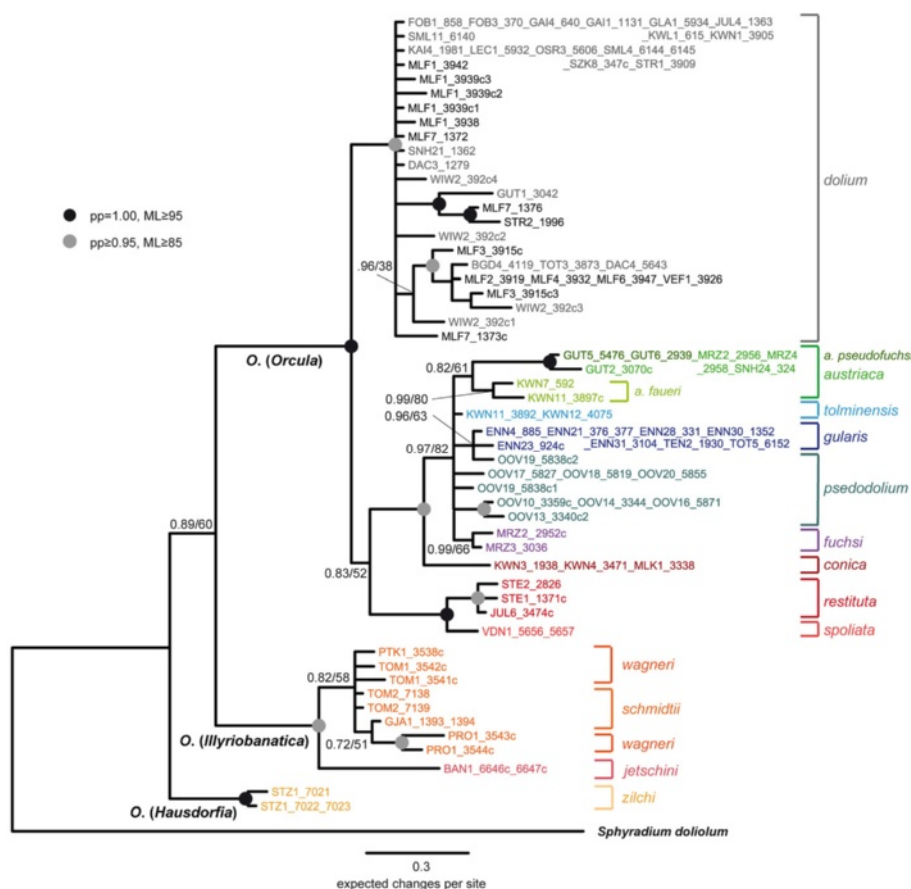
45

Figure 4 BI tree of the nc *H4/H3* sequences. Posterior probabilities and ML bootstrap values are provided for all nodes above species level. The scale bar indicates the expected number of substitutions per site according to the model of sequence evolution applied. The black and grey dots indicate nodes with high BI posterior probabilities and ML bootstrap values (see Figure). The colors of the specimen labels correspond to those used in Figures 3, 7 and 9.

estimated to 12.2 mya (14.8 to 9.5 mya, 95% HPD interval). The split between the subgenera *Orcula* and *Hausdorfia* (node II) was estimated to 9.9 mya (12.6 to 7.1 mya, 95% HPD), and the date of divergence of *O. dolium* from the other eight Alpine species (node III) was estimated to 8.5 mya (10.9 to 6.0 mya, 95% HPD). (2) In the second approach (Additional file 3), the divergence date of the outgroup taxa *O. bulgarica* and *O. aragonica* (node XVI) was calibrated to the time of the first occurrence of ancestral *O. aragonica* in the fossil record of the Iberian Peninsula. The resulting estimated mean node ages were 13.4 mya (23.5 to 4.7 mya, 95% HPD) for node I, 11.0 mya (19.4 to 3.9 mya, 95% HPD) for node II, and 9.4 mya (16.6 to 3.4 mya, 95% HPD) for node III, respectively. (3) The third approach (Figure 5) was a combination of the first two and included the calibration of two nodes (XIV and XVI). The estimated mean node ages were 12.4 mya (14.9 to 9.8 mya, 95% HPD) for node I, 10.2 mya (12.9 to 7.5 mya, 95% HPD) for node II, and 8.7 mya (11.2 to 6.3 mya, 95% HPD) for node III, respectively.

The node ages are largely congruent in approaches 1 and 2, suggesting that the two different calibration points did not produce conflicting results. However, the resulting 95% HPD intervals are extremely large when only the split between the outgroup taxa *O. bulgarica* and *O. aragonica* (node XVI; approach 2) is calibrated (Additional file 3). In approach 3 (two node datings) (Figure 5), ranges are similar as in approach 1 (Additional file 2), indicating that the 95% HPD ranges are mainly influenced by placing a prior on the stem of the tree.

The reconstruction of geographic ranges was performed with Lagrange [26], using the linearized maximum clade credibility tree inferred with approach 3 (two dating points). The distribution area of the genus *Orcula* was classified into seven geographic mountain areas, and the ancestral lineages were allowed to occupy a maximum of two ranges at the same time: In the constrained model (1), migration was prohibited between very distant areas, because some ancestral ranges were biogeographically extremely unlikely, and different migration probabilities were assigned

Figure 5 (See legend on next page.)
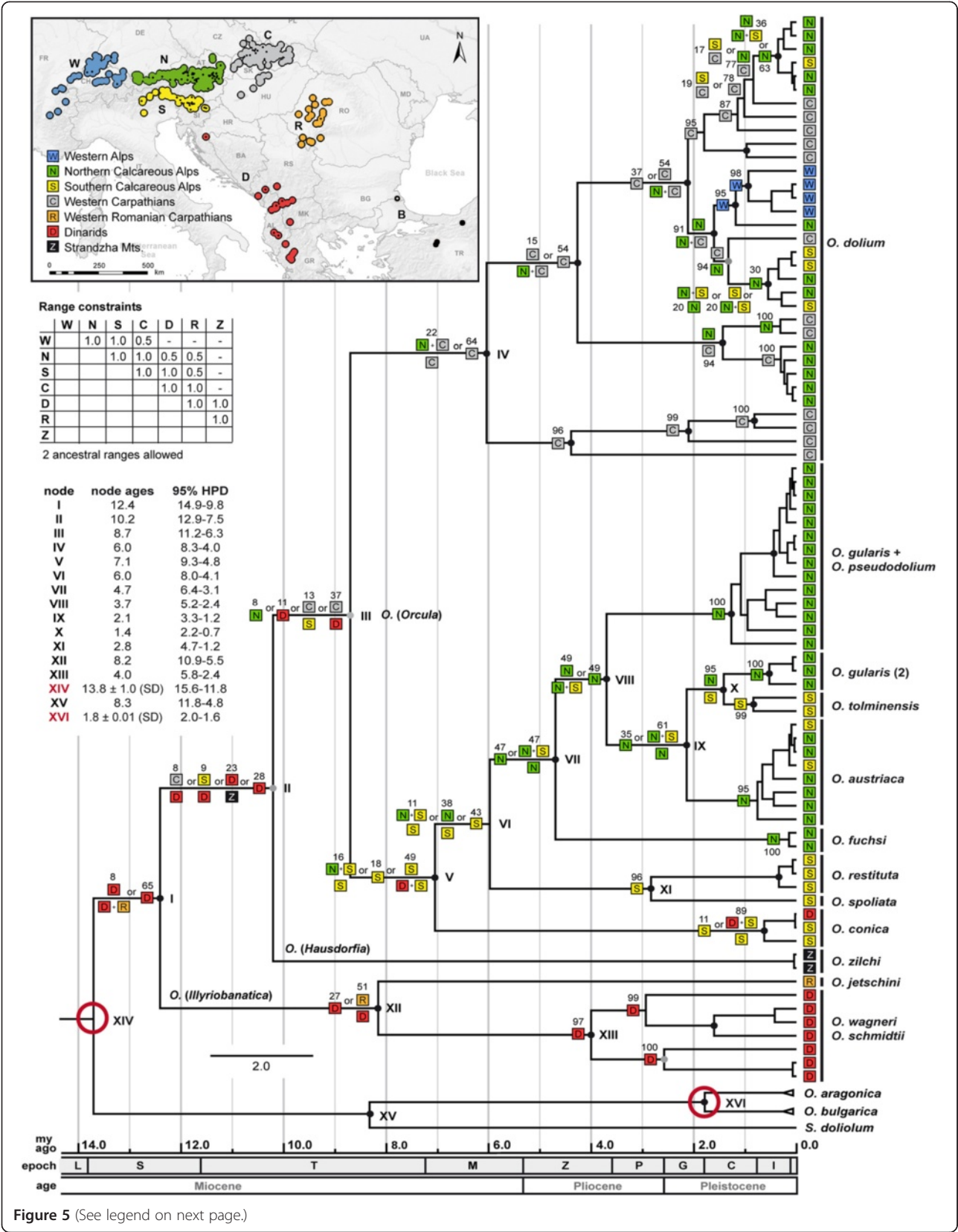
(See figure on previous page.)
Figure 5 Reconstruction of the historic geographic ranges. The map shows the distribution areas of the genus *Orcula* in seven geographic areas: Western Alps, Northern Calcareous Alps, Southern Calcareous Alps, Western Carpathians, Western Romanian Carpathians, Dinarides and western Black Sea region. The sampling localities are indicated by black dots. The collection sites of the outgroup specimens *O. aragonica* in Spain (Baetic system) and *O. bulgarica* in Turkey (Anatolia) are not shown in the map. The linearized molecular clock dated maximum-clade-credibility tree shows the relationships of selected mt lineages (concatenated *16S, 12S* and *COI sequences*). Black dots indicate nodes with high posterior probabilities (see Figure). The colored symbols at the branch tips indicate the geographic origin of each haplotype. At the cladogenesis events (nodes), all alternative ancestral subdivision/inheritance scenarios with likelihoods of 10% or more are indicated, separated by an "or", together with the respective likelihoods in%. When scenarios for cladogenesis events involve two ancestral areas, the symbol for the likely ancestral area/-s is/are provided left to each of the two branches. For nodes representing major splits, node ages and 95% posterior HPD intervals are indicated (see table). A time scale in mya is given below the tree. Abbreviations of the geological epochs: C: Chattian, A: Aquitanian, B: Burdigalian, L: Langhian, S: Serravallian, T: Tortonian, M: Messinian, Z: Zanclean, P: Piacenzian, G: Gelasian and C: Calabrian.

between adjacent and not immediately adjacent areas (Figure 5). In the unconstrained model (2), migration was permitted between all areas and with the same dispersal probabilities (Additional file 4).

The results are largely congruent at the outer branches of the trees, but the ancestral ranges and likelihoods estimated for the basal nodes/branches differ strongly between the two approaches. In the constrained model, the most recent common ancestor of the genus *Orcula* (node I) was likely distributed in the Dinarids (65%), and the ancestor of the subgenera *Orcula* and *Hausdorfia* (node II) was most likely distributed in the Dinarids (28%) or in an area additionally including the Bulgarian Strandzha Mts (23%) (Figure 5). The ancestor of the subgenus *Orcula* (node III) was most likely distributed in the Western Carpathians and the Dinarids (37%), the Western Carpathians and the Southern Calcareous Alps (13%), or in the Dinarids (11%). In the unconstrained model, a Dinarid ancestry of the genus is the most likely scenario as well, but with a lower probability (25%); an alternative range additionally includes the Northern Calcareous Alps (12%). Accordingly, the common ancestor of the subgenera *Orcula* and *Hausdorfia* (node II) was most likely distributed in either the Northern Calcareous Alps (16%) or in the Dinarids (11%). The unconstrained model also predicts different ranges for the common ancestor of the subgenus *Orcula* (node III): Northern Calcareous Alps (25%), Western Carpathians and the Southern Calcareous Alps (19%), or Western Carpathians and the Dinarids (15%).

The reconstruction of the geographic range history indicates that migrations to geographically distant mountain ranges represented rare events - the Western Black sea area, the Western Alps and the Western Romanian Carpathians were probably colonized only once. The Dinarides were probably re-colonized only once from the Southern Calcareous Alps during the Late Pleistocene or Holocene, namely by *O. conica*. However, in the subgenus *Orcula* migrations between Southern and Northern Calcareous Alps seem to have happened several times. Most complex patterns were found in *O. dolium*, which probably originated in the Western Carpathians and is

now found in most limestone areas of the Alps. The results suggest that the species migrated repeatedly between the Western Carpathians and the Alps, and colonized the Western Alps probably only once.

Genetic distances

In the mt data set, distances measured between the subgenera and species are extremely high (Table 2; Additional files 5 and 6). The uncorrected mean *p*-distances between the subgenera *Orcula* and *Illyriobanatica* are 28.6 (*12S*), 22.0 (*16S*) and 21.1% (*COI*), respectively. The mean distances between *Orcula* and *Hausdorfia* are 28.1 (*12S*), 25.2 (*16S*) and 24.7% (*COI*), whereas mean distances between *Illyriobanatica* and *Hausdorfia* are 31.4 (*12S*), 25.6 (*16S*) and 24.0% (*COI*), respectively. Surprisingly, the latter distances are even higher than the average distances between the genus *Orcula* and the outgroup *S. doliolum*, which are 30.9 (*12S*), 25.6 (*16S*) and 22.1% (*COI*). The species providing the largest intraspecific distances in the mt genes is *O. dolium* with 15.6 (*12S*), 14.0 (*16S*) and 18.3% (*COI*), followed by the *O. wagneri/schmidtii* complex with 12.3 (*12S*), 11.6 (*16S*) and 15.5% (*COI*). Within the group of Alpine endemics, highest intraspecific distances are observed in *O. tolminensis* with 3.7 (*12S*), 4.8 (*16S*) and 4.9% (*COI*), in the clade comprising *O. gularis* and *O. pseudodolium* with 4.3 (*12S*), 3.9 (*16S*) and 4.6% (*COI*), and in *O. austriaca* with 1.5 (*12S*), 1.9 (*16S*) and 3.6% (*COI*), respectively. Haplotype and nucleotide diversities calculated for the separate species clades with the complete *COI* data set are high in all groups (Table 3). The sequence divergences are also high within the set of *H4/H3* sequences (Additional file 7). The mean *p*-distances are 4.3% (*H4*: 1.6; *H3*: 2.1; *spacer*: 8.5%) between *Orcula* and *Illyriobanatica*, and 3.7% (*H4*: 1.4; *H3*: 2.4; *spacer*: 7.2%) between *Orcula* and *Hausdorfia*. Contrary to the pattern in the combined mt trees, the branch lengths of *Illyriobanatica* and *Hausdorfia* are shorter in the tree calculated with the nc sequences (Figure 4), resulting in a considerably lower mean distance of 2.9% (*H4*: 1.6; *H3*: 2.1; *spacer*: 5.1%) between the two subgenera. Mean distances of the

Table 2 Primer sets

Region	Primer (5' to 3')	Origin	Fragment size	T°C RocheTaq
COI fwd	COIfolmerFw: GGTCAACAATCATAAAGATATTGG	[54]	655 bp	50°C
COI rev	H2198-Alb: TATACTTCAGGATGACCAAAAAATC	[55]		
12S fwd	*12S*GastFw: TTACCTTTTGCATAATGGTTAAACTA	[56]	669 - 725 bp	54°C
12S rev	*12S*GastRv: CGGTCTGAACTCAGATCATG	[56]		
16S fwd	*16S*LOrc1_fwd: TTACCTTTTGCATAATGGTTAAACTA	[19]	838 - 890 bp	54°C
	*16S*LOrc2_fwd: TTACCTTTTGCATAATGGTTAAATTA	[19]		
16S rev	*16S*LOrc_rev: CGGTCTGAACTCAGATCATG	[19]		
H4/H3 fwd	Orc*H4*_left1: GTGCGTCCCTGGCGCTTCA	[19]	848 - 1095 bp	57°C
	Orc*H4*_left2: GGCGCTTCAGGGCGTACACT	[19]		
H4/H3 rev	Orc*H3*_right1: TGGGCATGATGGTGACACGCT	[56]		Finnzymes
				Phusion: 71°C
intern fwd	Orc*H4S*_left3: CGGTCTGAACTCAGATCATG	[19]		
intern rev	Orc*H3S*_right3: CGGTCTGAACTCAGATCATG	[19]		

Primer sequences for amplification and sequencing of the *COI, 12S, 16S* and *H4/H3* fragments. The *H4/H3* fragments were amplified with Finnzymes Phusion polymerase, wherefore the respective annealing temperatures are provided in addition.

three *Orcula* subgenera towards the outgroup *S. doliolum* are 9.7% (*H4*: 2.8; *H3*: 6.4; *spacer*: 20.0%).

Mitochondrial diversity in the subgenus *Orcula*

The *Orcula* species endemic to the Alps and the Alpine-Dinarid *O. conica* constituted more than three quarters of the samples analyzed and were in the focus of the present study. In order to display distributional patterns of the mt *COI* haplotypes, Median joining networks were calculated (Figure 6). The *COI* network corresponding to the *O. gularis/O. pseudodolium* clade in the mt trees (Figure 3, Additional file 1) comprises the majority of sequences (132) (Figure 6A1). Three specimens of *O. gularis* from the Northern Calcareous Alps (Ennstal Alps), clustering with *O. tolminensis* in the mt trees, are shown as a separate small network (Figure 6A2) because they are too diverged. Alternative scenarios explaining the relation of *O. gularis* and *O. pseudodolium* are discussed in detail in the following section. The main network of *O. gularis/O. pseudodolium* (Figure 6A1) is roughly divided into two clusters of haplotypes: the first includes samples of *O. gularis* from the Southern Calcareous Alps (Karawanks) and the Northern Calcareous Alps (Ennstal Alps, Dachstein, Ybbstal Alps and Totes Gebirge), and of a single specimen of *O. pseudodolium* (Northern Calcareous Alps: Upper Austrian Prealps). The second cluster includes all samples of *O. pseudodolium* (Northern Calcareous Alps: Upper Austrian Prealps), as well as a few haplotypes of *O. gularis* specimens from both the Northern (Salzkammergut Mts., Tennen Mts. and Totes Gebirge) and the Southern Calcareous Alps (Karawanks). The existence of Southern Calcareous Alpine haplotypes in both clusters indicates at least two independent migration events between the Southern Calcareous Alps and

the Northern Calcareous Alpine populations. Since the Southern Calcareous Alpine haplotypes in the second cluster are highly derived, it can be assumed that *O. gularis* was already present in that area for a longer period, probably before the LGM (30–18 kya; [4]). However, the similarity of the Southern and Northern Calcareous Alpine haplotypes in the first cluster implies a second, recent migration event from the latter area to the Southern Calcareous Alps. A second clade, shown in a separate network, is formed by three specimens of *O. gularis* from the Northern Calcareous Alps (Ennstal Alps).

A further network (Figure 6B) comprises all 56 samples of *O. austriaca*, including sequences of the three subspecies *O. a. pseudofuchsi* Klemm, 1967, *O. a. faueri* Klemm, 1967 and *O. a. goelleri* Gittenberger, 1978. The haplotypes of *O. a. faueri* (Southern Calcareous Alps: Karawanks) are embedded within a larger variety of haplotypes of the Northern Calcareous Alpine populations of *O. austriaca*. They are separated only by a single mutational step from haplotypes present in the Gutenstein Alps. The haplotypes of *O. a pseudofuchsi* (Northern Calcareous Alps: Gutenstein Alps) are located in two different clusters of the network, one with haplotypes of the Southern Calcareous Alpine *O. a. faueri*, the second with haplotypes of a neighboring Northern Calcareous Alpine mountain area (Rax-Schneeberg). The haplotypes of *O. a. goelleri* (Mürzsteg Alps) also show up in two different clusters of the network. Concluding, none of the three subspecies of *O. austriaca* is clearly delimited from the nominate form in its mt *COI* sequences.

The networks representing *O. fuchsi* (Figure 6E) and the Southern Calcareous Alpine endemics *O. conica* (Figure 6D), *O. spoliata* and *O. restituta* (Figure 6C)

Table 3 Genetic diversity and *p*-distances in the *COI* data set

species	sequence no.	haplotype no. (h)	haplotype div. (Hd)	nucleotide div. (Pi)	mean dist.	max dist.	1	2	3	4	5	6	7	8	9	10	11	12
O. dolium	35	33	0.997	0.107	10.4	18.3												
O. gularis/pseudodolium	129	74	0.974	0.028	2.9	5.7	20.2											
O. gularis (2)	3	2	0.667	0.01	1.5	1.5	18.5	16.5										
O. tolminensis	6	5	0.933	0.031	3	4.9	19	16.1	5.5									
O. austriaca	56	26	0.959	0.017	1.6	3.8	17.6	16.6	6.2	7.9								
O. fuchsi	10	5	0.822	0.005	0.5	0.8	18.5	19.7	15.5	15.5	13.6							
O. restituta	8	3	0.75	0.003	0.4	0.6	16.9	20.1	15.2	13.8	13.7	15.2						
O. spoliata	4	1	-	-	-	-	18.4	18.8	16.1	15.5	15	16.6	10.1					
O. conica	19	4	0.509	0.011	1.2	2.3	18.3	19.9	16.4	16.1	16.1	17.2	14.3	14.9				
O. schmidtii/wagneri	9	7	0.944	0.107	11.3	14.8	21.1	22.5	20.8	20.5	19.1	20.8	18.6	19.9	18.9			
O. jetschini	2	1	-	-	-	-	21.6	23.2	22.6	22.1	21	24	19.8	20.3	19.4	20.1		
O. zilchi	3	2	0.067	0.099	0.2	0.2	24.9	23.4	26.1	25.5	26.4	26.6	21.7	21.8	23.6	24.2	22.3	
S. doliolum	35	33	0.997	0.107	-	-	21.2	19.8	19.4	19.4	19.4	22	18.3	18.8	20.1	21.9	21.6	24.2
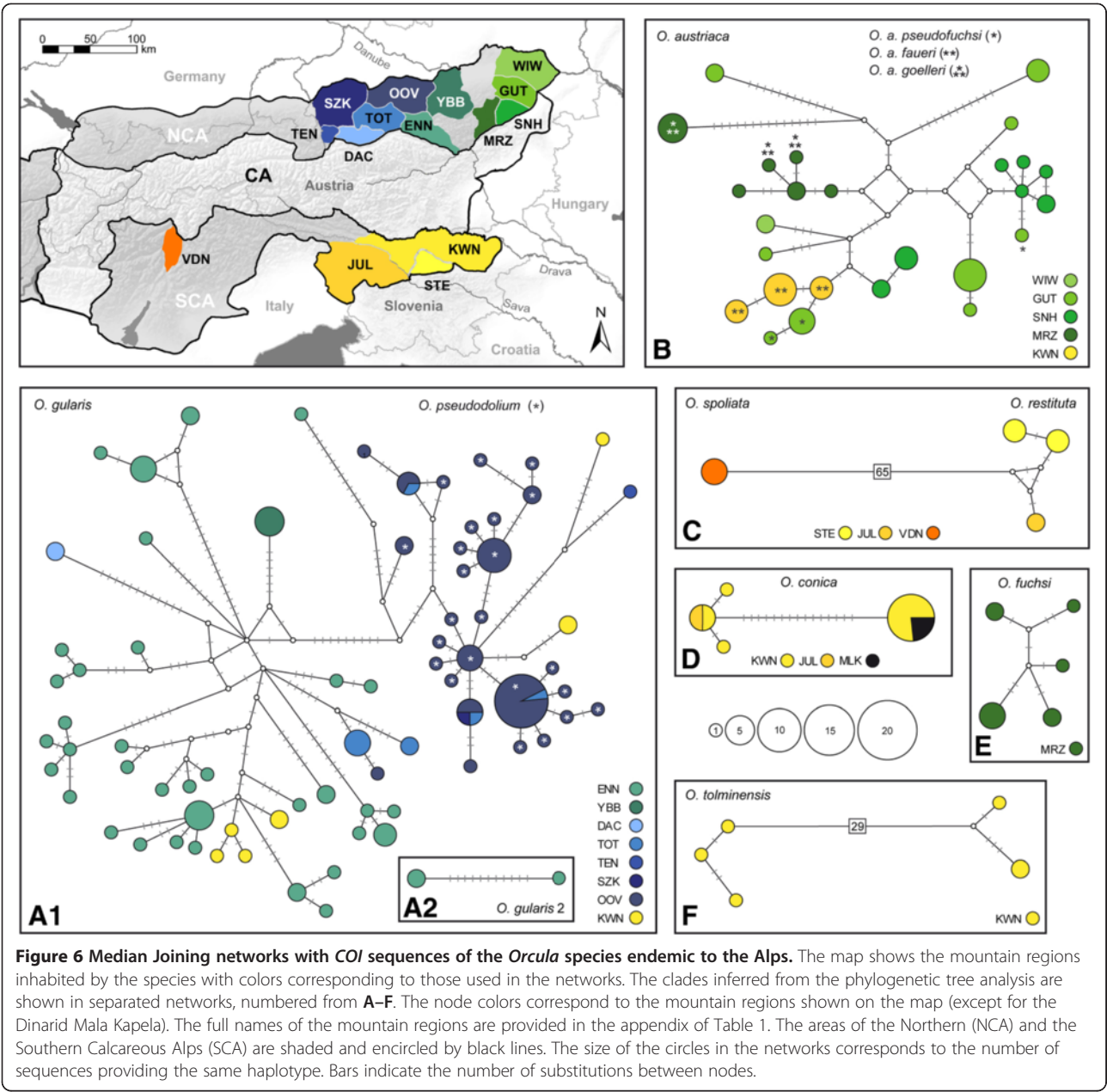
Figure 6 Median Joining networks with *COI* sequences of the *Orcula* species endemic to the Alps. The map shows the mountain regions inhabited by the species with colors corresponding to those used in the networks. The clades inferred from the phylogenetic tree analysis are shown in separated networks, numbered from **A–F**. The node colors correspond to the mountain regions shown on the map (except for the Dinarid Mala Kapela). The full names of the mountain regions are provided in the appendix of Table 1. The areas of the Northern (NCA) and the Southern Calcareous Alps (SCA) are shaded and encircled by black lines. The size of the circles in the networks corresponds to the number of sequences providing the same haplotype. Bars indicate the number of substitutions between nodes.

are less complex. The sequences of the latter two species are shown in a combined network because we found only one haplotype in *O. spoliata*. Noticeable is that the Dinarid (Mala Kapela, Croatia) specimens of *O. conica* feature a haplotype which was found also in the Southern Calcareous Alps (Karawanks), more than 200 km south-east, indicating a recent long distance migration event. The network of *O. tolminensis* (Figure 6F) shows two highly diverged sequence clusters, separated by 29 mutational steps. The pattern might be the result of a long evolutionary history in the Southern Calcareous Alps.

Morphological variation in the subgenus *Orcula*

The morphometric analysis was performed with the landmark data of the Alpine *Orcula* species (except for *O. dolium*) to evaluate the amount of morphological differentiation between the species and subspecies, respectively. The high intraspecific variability of shell morphs, even from the same localities, complicates a clear separation of the different species based on data of single specimens only. However, a higher resolution is obtained when mean shapes of several specimens per locality are compared with each other (Figure 7). In the Linear Discriminant Analysis (LDA) including
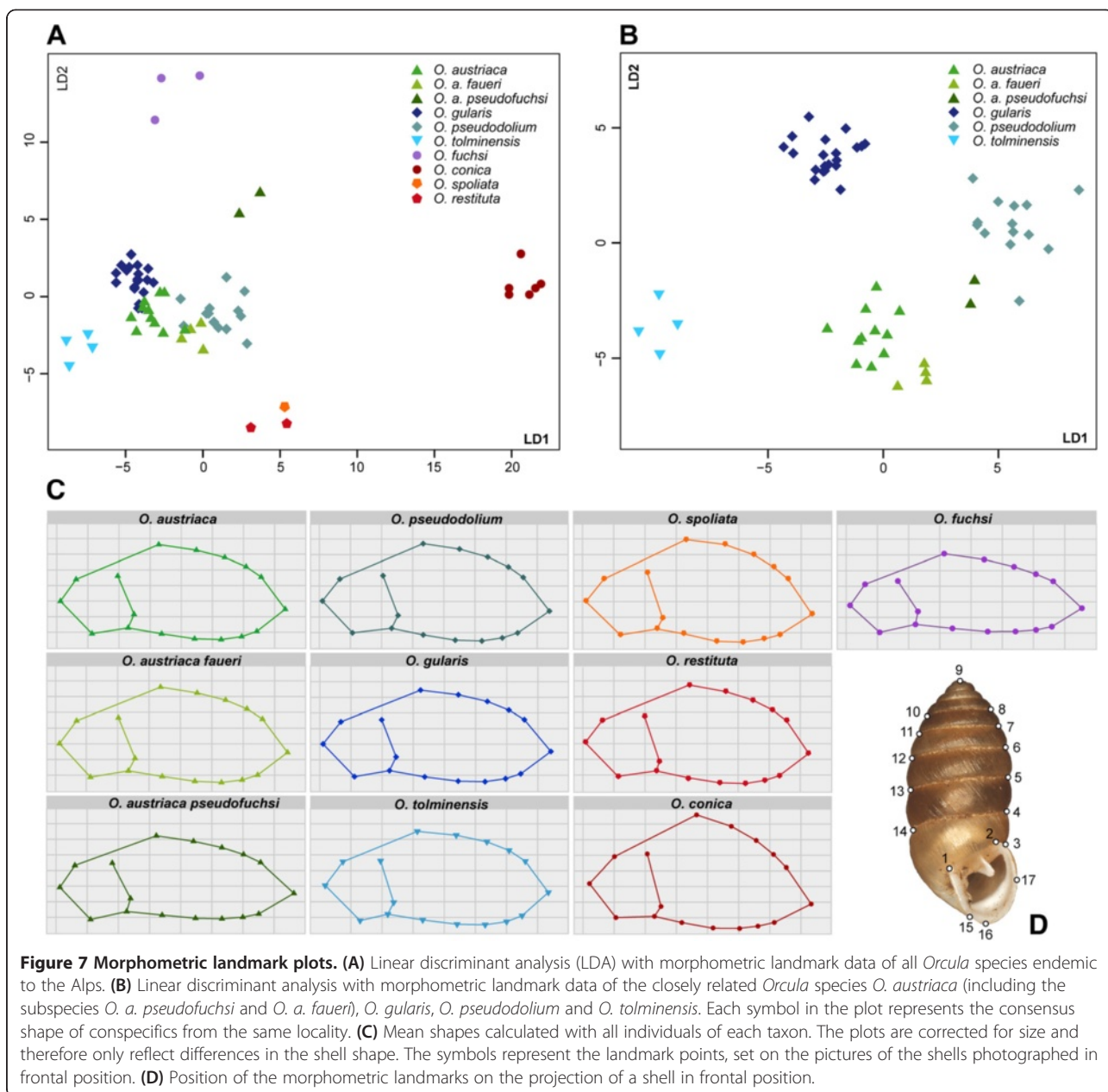
Figure 7 Morphometric landmark plots. (A) Linear discriminant analysis (LDA) with morphometric landmark data of all *Orcula* species endemic to the Alps. **(B)** Linear discriminant analysis with morphometric landmark data of the closely related *Orcula* species *O. austriaca* (including the subspecies *O. a. pseudofuchsi* and *O. a. faueri*), *O. gularis*, *O. pseudodolium* and *O. tolminensis*. Each symbol in the plot represents the consensus shape of conspecifics from the same locality. **(C)** Mean shapes calculated with all individuals of each taxon. The plots are corrected for size and therefore only reflect differences in the shell shape. The symbols represent the landmark points, set on the pictures of the shells photographed in frontal position. **(D)** Position of the morphometric landmarks on the projection of a shell in frontal position.

the mean shapes of all eight species, 85.02% of the total variance were explained by the first three discriminants (LD1: 59.37%, LD2: 16.09% and LD3: 9.56%; Figure 7A). In the LDA, shell shapes of *O. conica*, *O. fuchsi* and *O. tolminensis* are clearly differentiated. A distinct cluster is formed by *O. restituta* and *O. spoliata,* whose shells strongly resemble each other. The shell shapes of *O. a. pseudofuchsi* are unique and differ clearly from those of the nominate form *O. a. austriaca*, while *O. a. faueri* is hardly differentiated. The shape clusters of *O. gularis*, *O. austriaca* and *O. pseudodolium* are slightly overlapping (Figure 7A). However, when only the landmark data of the latter taxa (and the closely related *O. tolminensis*) are compared, the

species form well-defined clusters (Figure 7B). In the respective LDA (Figure 7B), 88.79% of the total variance is explained by the first three discriminants (LD1: 42.33%, LD2: 28.38% and LD3: 18.08%).

Discussion

Phylogeny and phylogeography of the genus *Orcula*

One of the main aims of this study was to clarify the phylogenetic relationships among *Orcula* species and to test whether the Alpine *Orcula* species represented a monophyletic group. Based on a comprehensive phylogenetic data set including samples of all 13 *Orcula* species there is clear evidence that the nine species distributed in

the Alps represent a monophyletic group corresponding to the subgenus *Orcula* as proposed by Páll-Gergely et al. [15]. Similarly, *Illyriobanatica* and *Hausdorfia* are each monophyletic in the mt and nc trees (Figures 3, 4). Previous considerations about the relationships of the *Orcula* species were made in particular by Gittenberger [17] and Schileyko [18], based on shell morphological and anatomical traits. Gittenberger's [17] suggestion that *O. gularis*, *O. tolminensis*, *O. pseudodolium* and *O. austriaca* are close relatives, demarcated from species representing rather independent lineages (*O. dolium*, *O. spoliata*, *O. restituta*, *O. conica* and *O. fuchsi*), was confirmed in the present study. In contrast, the phylogenetic scheme proposed by Schileyko [18] is not consistent with our results and would result in paraphyletic species complexes.

The reconstruction of the geographic range history supports a scenario in which the genus *Orcula* originated in the Dinarides during the Middle Miocene (Figure 5). Thus, despite the fact that nine of 13 extant *Orcula* species are distributed in the Alps, the area was most likely not the center of origin, but was colonized from the Dinarids. The lineages of the three subgenera most likely split during the Middle and Late Miocene - during that time period, Alps, Dinarides and Carpathians were partly separated by a lateral branch of the Mediterranean Sea [27]. The separation of *O. dolium* from the group including the eight Alpine endemics (and the Alpine-Dinarid *O. conica*) was dated in the Late Miocene and could be explained with the formation of Lake Pannon, which separated Eastern Alps and Western Carpathians during the Tortonian and reached its maximum extent about 10 mya [28]. The results suggest that *O. dolium* originated in the Western Carpathians and colonized the Eastern Alps first during the Pliocene. The radiation into numerous mt lineages during the Pleistocene can be explained with divergence in separated glacial refuges as suggested by Harl et al. (2014) [19]. The diversification of the other *Orcula* species endemic to the Alps (including the Alpine-Dinarid *O. conica*) probably started shortly after the split from *O. dolium*, during the Late Miocene or the Lower Pliocene. Accordingly, the diversification of most Alpine *Orcula* species pre-dated the Pleistocene and their speciation cannot plausibly be explained solely by divergence in separate glacial refuges as proposed by Zimmermann (1932) [20] - only the split of the closely related *O. austriaca*, *O. tolminensis* and *O. gularis* is dated in the Pleistocene (Figure 5).

The species of the subgenus *Illyriobanatica* are distributed in the Dinarides and the Western Romanian Carpathians. Several other land snail taxa share similar distribution patterns and inhabit both mountain ranges, for instance the hygromiid *Xerocampylaea zelebori* (L. Pfeiffer, 1853), the aciculid *Platyla wilhelmi* (A. J. Wagner, 1910) and the clausiliid genus *Herilla* Adams & Adams, 1855 [29]. Our data suggest that the Dinarid *O. wagneri*/*schmidtii* complex and

the Western Romanian *O. jetschini* split during the Late Miocene. The formation of Lake Pannon could have influenced their separation as well - parts of the Western Romanian Carpathians (including the current distribution area of *O. jetschini*) formed islands during the Middle Tortonian [28,30].

Summarizing, the reconstruction of the geographic range history indicates that the separation of the major groups within the genus *Orcula* was linked to palaeogeographic events which shaped Europe during the Miocene. The patchy distribution of limestone rock certainly constituted an important factor in the diversification of lineages because all *Orcula* species are more or less calciphilous. Since lowland areas like the Pannonian Basin featured almost no limestone rock, active migration between mountain ranges was probably hampered. The Pleistocene glaciations obviously had a strong impact on the current distributions of the Alpine endemics because their areas are all located near the eastern margins of the LGM glacier line. However, most of the latter species presumably diverged from each other in the Late Miocene and the Pliocene already, and not during the Pleistocene (Figure 5).

Hybridization within the subgenus *Orcula*

In most of the species of the subgenus *Orcula*, coherent mt and nc sequence patterns (Figures 3, 4, 6) as well as common morphological traits (Figure 7) were found. Thus, these species seem to be reproductively isolated from each other. Moreover, several species were found to occur sympatrically without indication for hybridizations: *O. austriaca* and *O. tolminensis*, *O. fuchsi* and *O. austriaca*, *O. gularis* and *O. conica*, *O. dolium* with *O. austriaca*, and *O. gularis* with *O. conica* and *O. fuchsi*. Nonetheless, our data strongly support that hybridization happened between *O. gularis* and *O. pseudodolium*. Although these two species could be discriminated by their shell forms in the morphometric analyses (Figures 7A and 7B) and provided different *H4/H3* sequences (except for a single specimen of *O. pseudodolium* from the potential hybridization area) (Figure 4), a clear assignment to one or the other species was not possible based on the mt sequences. Most mt haplotypes of *O. gularis* cluster with *O. pseudodolium* (Figures 3, 6), and only three single specimens of *O. gularis* (ENN21_377, ENN23_924 and ENN4_885) from the Ennstal Alps (Austria, Styria) show distinct mt variants, which form a sister clade of *O. tolminensis*. Specimens from four sites (OOV17, OOV18, OOV19, OOV20) even show transitional states in the expression of the palatal folds (from a backward orientated tooth to a diagonal bulge) (Figure 8), indicating recent hybridization.

Potential causes of non-monophyly of species in mt trees theoretically can be inferred from the depth of the

Figure 8 Pictures of *O. gularis* and *O. pseudodolium* specimens.
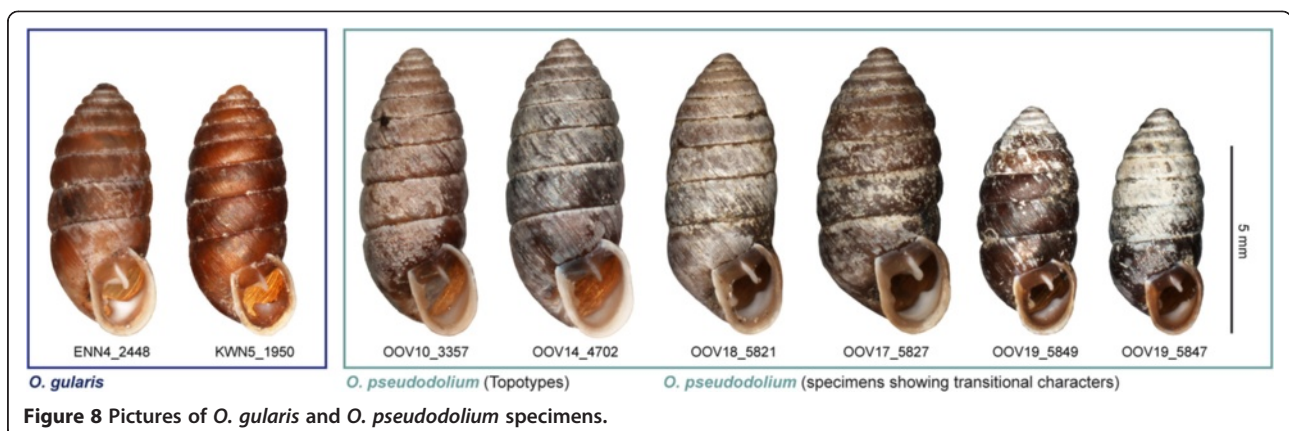
coalescences in gene trees, geographical distribution of shared genetic markers, and concordance with results of admixture analyses of nuclear multilocus markers. [31]. In land snail species, incomplete lineage is discussed in species of the hygromiid genus *Xerocrassa* Monterosato, 1892 [32] and the helicid *Cornu aspersum* [33], whereas mt introgression is assumed to have happened in other *Xerocrassa* species [31] and in the camaenid genus *Euhadra* Pilsbry, 1890 [34].

The non-monophyly of *O. gularis* and *O. pseudodolium* in the mt trees might be explained by three different scenarios at least: (1) *O. gularis* acquired its mitochondria from *O. pseudodolium* by mt introgression, but genuine mt variants of *O. gularis* still exist in the population of the Ennstal Alps. (2) *O. pseudodolium* acquired its mitochondria from *O. gularis* by mt introgression, and the aberrant mt variants in the three *O. gularis* specimens of the Ennstal Alps were acquired from *O. tolminensis* by mt introgression. (3) The mixed mt patterns in the mt *O. gularis*/*O. pseudodolium* clade resulted from incomplete lineage sorting. Scenario 1 would require hybridization between *O. gularis* and *O. pseudodolium* only and, thus, provide a more parsimonious explanation than scenario 2. A close relationship between *O. austriaca*, *O. gularis* and *O. tolminensis* is also supported by similarities in the genital anatomy, whereas *O. pseudodolium* shows quite distinct traits [17]. Furthermore, *O. gularis* and *O. tolminensis* strongly resemble each other in their aperture traits by the unique presence of a palatal tooth. Incomplete lineage sorting (scenario 3) would not explain the relation between *O. pseudodolium* and *O. gularis* sufficiently. The mt sequence patterns in the network (Figure 6A) rather indicate that hybridization occurred at different points of time - some haplotypes of *O. gularis* are highly diverged from those of *O. pseudodolium* whereas others are identical or differ only by a few substitutions from each other. Moreover, the existence of the second *O. gularis* clade in the Ennstal Alps virtually cannot be explained by incomplete lineage sorting

(Figure 3). Shedding more light on this topic would require additional sampling the potential hybridization area and analyzing (additional) nc markers from a larger number of specimens.

Another issue addressed in our study is the morphological variability within *O. austriaca*. Apart from the common form, three subspecies were described for *O. austriaca*. Among those, *Orcula a. pseudofuchsi* is of special interest because its shells are more elongated than those of the nominate form of *O. austriaca*. Klemm [35] hypothesized that *O. a. pseudofuchsi* represents an 'intermediate' between *O. a. austriaca* and *O. fuchsi*, or descended from the same common ancestor at least. Despite unequivocal shell morphological differences between specimens of *O. a. austriaca* and *O. a. pseudofuchsi* (Figure 7), the two taxa could not be delimitated by the mt and nc markers analyzed (Figures 3 and 4). Moreover, no intermediates between *O. austriaca* and *O. fuchsi* were found at Mt. Göller (Lower Austria) where both species co-occur and, in contrast to the assumption of Klemm [35], the two species were not even sister species (in the mt trees). Hence, the aberrant shell shape of *O. a. pseudofuchsi* is most likely not the result of hybridization but rather evolved uniquely in the population of *O. austriaca* from Mt. Gösing (Lower Austria). Similarly as in *O. austriaca*, conspecific populations strongly differing in shell morphology but not in their mtDNA were observed in the Western Carpathian populations of *O. dolium*. *Orcula dolium brancsikii* Clessin, 1887, exhibiting strongly elongated shells, was found next to populations with specimens featuring rather globulous shells, but a distinction of the two forms was not possible with the nc and mt markers used [19]. Another subspecies, *O. a. faueri*, inhabits the Southern Calcareous Alps, geographically separated from the nominate form of the Northern Calcareous Alps. Despite the geographic distance, the mt haplotypes of *O. a. faueri* are embedded within the diversity of the Northern Calcareous Alpine population, suggesting that *O. austriaca* colonized the Southern Calcareous Alps very

recently (Figure 6). In contrast *O. a. faueri* could not be differentiated from the common form by its shell morphology (Figure 7). Only in the nc *H4/H3* trees there is a comparably strong bifurcation between the lineages of the Northern Calcareous Alps and the Southern Calcareous Alps, indicating that the evolutionary history of the species is probably more complex (Figure 4). A possible explanation could be that the populations of the Northern Calcareous Alps and the Southern Calcareous Alps diverged in allopatry, but intermixed recently, leading to mt capture and the loss of the genuine mt variants of *O. a. faueri*.

Glacial refuges of the *Orcula* species endemic to the Alps

Although the diversification of the *Orcula* species endemic to the Alps probably started already in the Late Miocene and can only partly be attributed to speciation in glacial refuges, the Pleistocene LGM (30–18 kya; [4]) and earlier glacial maxima obviously affected the current distribution to a great extent. Since all of the latter species are strictly calciphilous, they probably could not survive the LGM in lowland areas surrounding the Alps like the closely related *O. dolium* [19]. However, most of the Alpine endemics show wide altitudinal ranges from the valleys up to high mountain areas (e.g., *O. gularis* from 400 to 2000 m asl) and are adapted to cold climates. Even though the LGM climatic snowline was 1000 to 1500 meters below the current level in peripheral ranges of the Eastern Alps [36], lower mountain ranges potentially provided suitable conditions during glacial periods. Moreover, the current distributions of all Alpine endemic *Orcula* species include areas not covered by ice during the LGM (Figure 9). The existence of Eastern Alpine refuges is also supported by several recent molecular genetic studies dealing with mountain plants [8,37,38] and invertebrates [9,10,39]. Besides, the Eastern Alpine margins harbor several endemic species with low active dispersal capabilities, among those blind troglobiotic beetles [40] and endemic land snail species restricted to high altitudes [41]. The genetic diversity within *Orcula* allows to conclude that the Alpine endemics outlasted the LGM and earlier Pleistocene cold stages probably in several smaller refuges at the periphery of both the Northern and the Southern Calcareous Alps and did not suffer from genetic bottlenecks.

Conclusions

The results of the present study indicate that the evolutionary history of the genus *Orcula* dates back to the Middle Miocene. The three subgenera most likely derived from an ancestor that was distributed in the Dinarides, and their separation can be explained by palaeogeographic events preventing migration between the mountain ranges populated. Although the Alps

were probably not the origin center of the genus, they gave birth to the majority of species. The structuring of the Alps, with two geographically separated major limestone areas (Southern and Northern Calcareous Alps), was of great importance for the diversification of the local *Orcula* species, most of them being strictly calciphilous. Within the group of Alpine endemics, most speciation events seem to predate the Pleistocene. Their current distribution patterns, however, were strongly shaped by the LGM glacier extent. Most taxa could be differentiated well by both morphologic and genetic traits, with the exception of *O. gularis* and *O. pseudodolium*. The latter two species differ in their shell morphology and nc DNA but cannot be distinguished by their mt DNA sequences, indicating mt introgression.

Methods

Study area and sampling

Samples of all 13 *Orcula* species were collected in the years 2007 to 2012 in ten different countries. Figure 1 shows the location of collection sites investigated. The distribution areas in Figure 1 are based on literature data [2,15,21,42], collection data of the Natural History Museum Vienna (NHMW) and the Senckenberg Museum (SMF), and data of the present study. Of a total of 115 localities investigated in the present study, most sites (101) were located in the Alps (Table 1). Elevation and position of the localities were determined via GPS. Samples were collected in various habitats covering an altitudinal range from 190 m to 2200 m above sea level (asl). One to four specimens of each species per site were prepared for the DNA sequence analyses and stored in 80% ethanol, following the protocol of Kruckenhauser et al. [43]. In addition, a number of empty shells were collected from the same sites for the morphometric analyses. DNA samples of the outgroup species *Orculella bulgarica* and *Orculella aragonica* were obtained from B. Gómez-Moliner (Universidad del País Vasco, Vitoria, Spain) and were already used by Arrébola et al. [44]. All other voucher specimens were deposited in the Natural History Museum Vienna (NHMW). In order to provide an overview of the taxa investigated, pictures of shells of selected type specimens are shown in Figure 2. The species and subspecies determination was based on a combination of shell characters (expression of aperture folds and shell form) and the geographic distributions reported in literature [2,21].

Outgroup selection for phylogenetic trees and fossil calibration

In the course of the investigations on *Orcula*, samples of most other orculid genera (including *Alvariella* Hausdorf, 1996, *Orculella* Steenberg, 1925, *Pilorcula* Germain, 1912, *Pagodulina* Clessin, 1872, *Sphyradium* Charpentier, 1837 and *Schileykula* Gittenberger, 1983) were analyzed for the
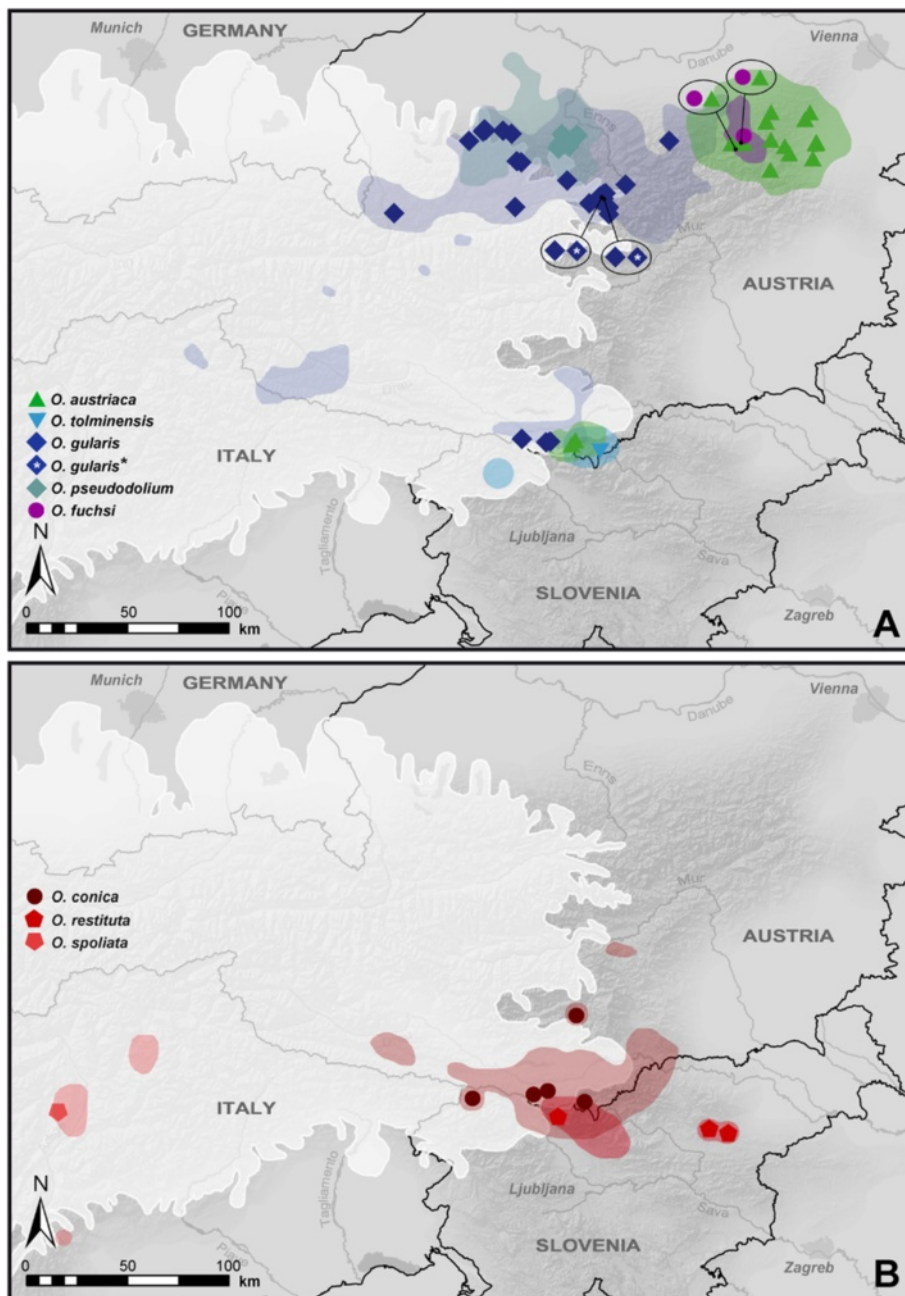
Figure 9 Distribution of mt clades of the *Orcula* species endemic to the Alps. As the distribution areas of five of the species overlap in the Southern Calcareous Alps, the data are displayed in two separate maps **(A and B)**. The colored symbols indicate the distribution of the mt clades, the color shaded areas represent the distribution areas of the respective *Orcula* species. In case that two species or species clades, respectively, were found at the same localities, the symbols are shown in ovals pointing towards the locality. The white shaded areas represent the glacier extent during the LGM (30–18 kya; [4]). The data on the maximum extent of glaciers during the LGM was published by [72], and modified by [8].

same set of mt and nc markers. Preliminary analyses based on this data set clearly support the monophyly of a group containing *Orcula*, *Schileykula*, *Sphyradium* and *Orculella*, with *Orcula* being the sister group to the other three genera (Harl et al. in prep.). Hence, all of these three genera represent equivalent outgroup taxa. We used the monotypic *Sphyradium doliolum* as outgroup for the

calculation of the mt (*COI*, *12S*, *16S*) and nc (*H4/H3*) phylograms (Figures 3, 4). Within the family Orculidae, *S. doliolum* is by far the most widespread species with an area extending from Western Europe to Kyrgyzstan [45]. *Orculella bulgarica* and *Orculella aragonica* were additionally included as outgroups in the molecular clock analyses. *O. bulgarica* is the most widespread

species within *Orculella*, distributed from southern Europe to Armenia [45], whereas *O. aragonica* is the only *Orculella* species of the Iberian Peninsula [46]. Their sister group relationship is supported by molecular genetic (*COI*, *16S*) and anatomical data [44], and a (within the genus uniquely) shared preference for wet habitats such as small marshes [46]. The paleontological record of *O. aragonica* comprises more than 30 Pliocene to Holocene sites, of which the Almenara-Casablanca karst complex (Castellón, Spain) features the oldest records (1.8 mya [47]). The site features a continuous Miocene to the Early Pleistocene fossil record, allowing to determine the species' first occurrence in that area fairly precisely. One of the assumptions in the molecular clock analyses was that this first record coincides with the colonization of the Iberian Peninsula by ancestral *O. aragonica* or the split between *O. aragonica* and *O. bulgarica*, respectively. The second assumption was that the fossil orculid species *Nordsieckula falkneri* represents the most recent common ancestor of *Orcula*, *Orculella*, *Sphyradium* and *Schileykula* Gittenberger, 1983 (not included in the present study). The species is known from Middle Miocene sediments in Gründlkofen bei Landshut (Germany) and Nowa Wieś Królewska (Poland) and was previously classified into the genus *Orcula* [48]. Based on strong morphological similarities *N. falkneri* is considered as closely related to the genera *Orcula*, *Orculella*, *Sphyradium* and *Schileykula* [1], but as a distinctive trait it features a tooth-like subangularis [49], which was yet solely found in some of the extant North-African *Orculella* species. Its only congeneric *Nordsieckula subconica* (Sandberger, 1858) is known from Late Oligocene (Hochheim, Germany) to Early Miocene (Tuchořice, Czech Republic) successions [50]. *N. subconica* features an additional trait, a third columellar lamella (infracolumellaris), which is neither present in *N. falkneri* nor in any extant species of the genera *Orcula*, *Schileykula*, *Sphyradium* and *Orculella* (Figure 10). Since *N. falkneri* temporally succeeds *N. subconica* and was found in the same area, it might be a direct descendant of the latter. In the molecular clock analyses, the basal node of the tree was dated with the latest record of *N. falkneri* (Nowa Wieś Królewska, Poland), which was classified to the Astaracian (MN6/7) [51,52], corresponding to a time period comprising the Middle Miocene Langhian (15.97 to 13.65 mya) and the Serravallian (13.65 to 11.61 mya) [53]. The node age was set to 13.79 mya (SD ±1.0), the mean age between the lower boundary of the Langhian and the upper boundary of the Serravallian.

PCR and sequencing

A total of 295 specimens were analyzed by means of molecular genetics (specimen numbers in brackets): *O. austriaca* (56), *O. conica* (19), *O. dolium* (35), *O. fuchsi*
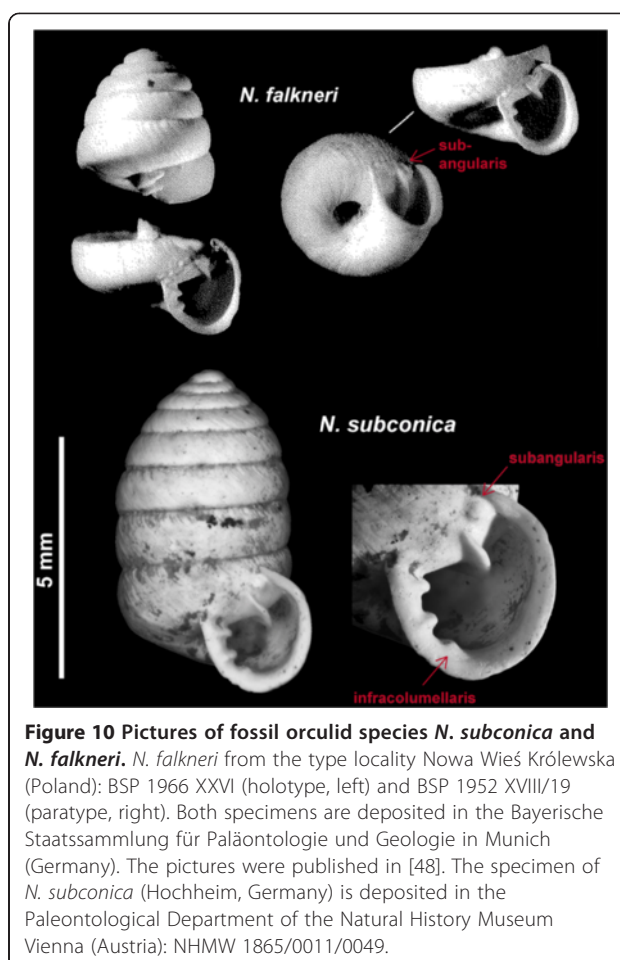

Figure 10 Pictures of fossil orculid species *N. subconica* and *N. falkneri*. *N. falkneri* from the type locality Nowa Wieś Królewska (Poland): BSP 1966 XXVI (holotype, left) and BSP 1952 XVIII/19 (paratype, right). Both specimens are deposited in the Bayerische Staatssammlung für Paläontologie und Geologie in Munich (Germany). The pictures were published in [48]. The specimen of *N. subconica* (Hochheim, Germany) is deposited in the Paleontological Department of the Natural History Museum Vienna (Austria): NHMW 1865/0011/0049.

(11), *O. gularis* (84), *O. jetschini* (2), *O. pseudodolium* (48), *O. restituta* (8), *O. spoliata* (4), *O. tolminensis* (6), *O. wagneri/schmidtii* (9), *O. zilchi* (3), *S. doliolum* (1), *O. bulgarica* (6) and *O. aragonica* (4). Unique labels, consisting of a specimen number and a locality tag, were assigned to every specimen. The latter is a three letter code, with each code representing a geographic mountain region (Table 1). DNA was extracted using the QIAgen Blood and Tissue Kit. A section of the mt *COI* (655 bp) was analyzed in all specimens with the primers COIfolmerFw [12] (modified from Folmer et al. [54]) and H2198-Alb [55]. As the genetic distances between the *Orcula* species were extremely high, deeper splits in the phylogenetic trees could not be resolved with the *COI* alone. Therefore, additional mt and nc markers were analyzed in a selection of 86 individuals of *Orcula* and the eleven outgroup specimens: sections of the mt *12S* (673 to 725 bp) and *16S* (838–890 bp), and a sequence region comprising large parts of the nc histone genes *H3* (345 bp) and *H4* (259 bp), and the intermediate non-coding spacer (244–490 bp). The *12S* primers (*12S*GastFw and *12S*GastRv) and the *H4*/*H3* reverse-primer (Orc*H3*Right1) were published in [56]. The *16S*

primers (*16S*LOrc1Fw, *16S*LOrc2Fw and *16S*LOrcRv) and the other *H4*/*H3* primers (Orc*H4*Left1, Orc*H4*Left2, Orc*H3*Sright3 and Orc*H4*SLeft3) were developed by [19]. For direct sequencing, DNA fragments were amplified with the RocheTaq© DNA polymerase with 3 mM MgCl₂. The PCR started with 3 min at 94℃, followed by 35 cycles with 30 s at 94℃, 30 s at the particular annealing temperatures (Table 3), 1 min at 72℃, and a final extension for 7 min at 72℃. Primer sequences and annealing temperatures are listed in Table 3. The *H4*/*H3* fragments were amplified with the primers Orc*H4*Left1 (or the alternative primer Orc*H4*Left2) and Orc*H3*Right1, but sequencing was performed with the internal primers Orc*H3*Sright3 and Orc*H4*SLeft3. Some specimens showed to be heterozygous regarding the non-coding spacer region. In those cases, the PCR was repeated with the proofreading Finnzymes Phusion© polymerase. The PCR started with 30 s at 98℃, followed by 35 cycles with 10 s at 98℃, 10 s at the particular annealing temperatures (Table 3), 30 s at 72℃, and a final extension for 7 min at 72℃. The PCR products were excised from 1% agarose gels and purified using the QIAquick© Gel Extraction Kit (QIAGEN), extended by A-endings with the DyNAzyme II© DNA polymerase (Finnzymes) and then cloned with the TOPO-TA© cloning kit (Invitrogen). Purification and sequencing (in both directions) was performed at LGC Genomics (Berlin, Germany) using the PCR primers, except for *H4*/*H3* (see above). The primer sequences are shown in Table 2. All sequences are deposited in GenBank under the accession numbers KM188500 - KM188950.

Sequence statistics and phylogenetic tree reconstruction

The raw sequences were edited manually using Bioedit v.7.1.3 [57]. The alignment of the *COI* sequences was straightforward since there were no insertions or deletions (indels). Median-Joining networks were calculated for the eight Alpine endemics with Network v.4.6.0.0 (Fluxus Technology Ltd.) applying the default settings. In order to reduce unnecessary median vectors the networks were then post-processed with the MP (Maximum parsimony) option.

BI and ML phylograms were calculated based on the concatenated alignments (*COI*, *12S* and *16S*) of 86 *Orcula* specimens and the outgroup *S. doliolum*. The subset included all single specimens of *O. wagneri/schmidtii*, *O. jetschini*, *O. zilchi* and *O. dolium*, as well as a selection of specimens from the Alpine endemics. The *12S* and *16S* sequences were aligned with ClustalX v.2 [58] and adjusted manually. Less conserved sequence regions were excluded by trimming the alignment with TrimAl v.1.3 [59]. The original *12S* alignment contained 790 bp of which all 271 gap sites were removed using the 'no gap' option (removal of all sites containing gaps). Another 67 positions were excluded by applying

the 'strict' option (trimming based on an automatically selected sequence similarity threshold [59]). The original *16S* alignment contained 945 positions of which 241 gap sites were excluded. Another 111 sites were removed with the 'strict' option. Subsequently, *COI*, *12S* and *16S* were concatenated and identical sequences were collapsed, resulting in a total of 86 unique haplotypes.

Substitution saturation was assessed for all single mt data sets using the test of [23], implemented in DAMBE v.5.2.78 [60]. In order to accommodate substitution saturation in the 3rd codon positions of the *COI* and to test the influence on the phylogeny, alternative trees were calculated excluding the third codon positions of the *COI*. Having collapsed identical sequences, the concatenated alignment of this data set contained a total of 77 haplotypes.

The optimal substitution models were determined for all individual data sets with JModelTest v.2.1.5 [61], based on the corrected Akaike Information Criterion (AICc). TrN + I + G was the best-fit substitution model for *COI*, *COI* '1st and 2nd codon positions' and *16S*, and TPM1uf + I + G for *12S*. The optimal model for the entire concatenated alignments was GTR + G + I. However, owing to the limited number of models applicable in MrBayes [62,63], the evolutionary model was set to GTR + G + I for all separate data partitions in the Bayesian analyses.

BI and ML phylograms were also calculated with the *H4*/*H3* data set. Similarly, the alignment was split into three partitions, namely *H4*, *spacer* and *H3*. The *spacer* was aligned with ClustalX v.2 [58] and all sites with gaps were removed. The data set contained 94 sequences (including additional clones when specimens were heterozygous for the *H4*/*H3*) of 87 specimens, which were collapsed to 53 unique haplotypes. Based on the AICc, the best fitting substitution models were K80 for *H3* (345 bp) and *H4* (259 bp), and K80 + G for the noncoding *spacer* (206 bp, gaps excluded). The optimal model for the concatenated *H4/H3* sequences was GTR + G.

The BI analyses were calculated using the concatenated (mt and nc) alignments, with three data partitions each, allowing MrBayes v.3.2.2 [62,63] to evaluate the model priors of each partition independently. Applying the respective model parameters, the analyses were run for 5x10⁶ generations each (2 runs each with 4 chains, one of which was heated), sampling every hundredth tree. Tracer v.1.5 [25] was used to assess whether the two runs had converged and when the stationary phase was reached, which was the case already after several thousand generations. In a conservative approach, the first 25% of trees were discarded as burnin and a 50% majority rule consensus tree was calculated from the remaining 37,500 trees.

ML bootstrap trees were calculated with MEGA v.5.1 [24], applying the models GTR + G + I to the mt and

GTR + G to the nc sequences, respectively, but without using separate data partitions (since this option is not supported by MEGA v.5.1). For all data sets, 500 bootstrap replicates were performed using Subtree-Pruning-Regrafting (SPR) as heuristic method for tree inference.

Based on the alignment including all *COI* sequences, calculations of mean *p*-distances between species clades and maximum *p*-distances within the clades were performed with MEGA v.5.1 [24]. For the *12S* and *16S* alignments (gap sites excluded), mean *p*-distances between the species clades were calculated, and for the *H4/H3* alignments mean *p*-distances were calculated between the subgenera only. Haplotype and nucleotide diversities were evaluated with DnaSP v.5.10 [64] for the complete *COI* data set.

The sequence alignments used for the calculation of the mt and nc trees are provided in Additional files 8 and 9.

Molecular clock analysis and reconstruction of geographic range history

The calculations of molecular clock dated linearized BI trees were performed in BEAST v.1.7.5 [25] with the concatenated mt sequences of *COI*, *12S* and *16S*. Apart from *S. doliolum* (1 specimen), 4 specimens of *O. bulgarica* and 6 specimens of *O. aragonica* were included as outgroup taxa, because these were used for dating the trees. The molecular clock analyses were calculated with the complete *COI* and the trimmed *12S* and *16S* alignments, following the same procedure as for the inference of the phylograms. Having removed all gap sites and performing the "strict" option in TrimAl v.1.3 [59], the *12S* and *16S* alignments contained 449 and 575 positions, respectively. For model selection and molecular clock analyses, identical sequences were collapsed to a total of 85 haplotypes (out of 97 specimens). The best fitting substitution models were calculated separately for each partition with JModeltest 2.1.5 [61], based on the AICc, resulting in the models HKY + G + I for *COI* and TN93 + G + I for *12S* and *16S*. The relative rate variation among lineages was tested separately for all three partitions with the molecular clock test implemented in MEGA v.5.1 [24] applying the optimal substitution models inferred with JModeltest 2.1.5. Since the null hypothesis of equal evolutionary rates throughout the trees were rejected at a 5% significance level (P =0), divergence times were estimated under a relaxed molecular-clock in all molecular clock analyses. The divergence times of the mitochondrial lineages were estimated using three different approaches: (1) The basal node of the tree was dated to 13.79 ± 1.0 (SD) mya (15.43 to 12.15 mya, 95% HPD interval), corresponding to the mean age between the lower boundary of the Langhian (15.97 mya) and the upper boundary of the Serravallian (11.61 mya) [53] or the Middle Miocene Astaracien,

respectively, the period to which the fossils of *N. falkneri* were dated (see chapter 'Outgroup selection for phylogenetic trees and fossil calibration') (Additional file 2). (2) The node marking the split between *O. bulgarica* and *O. aragonica* was dated with the presumed first appearance of ancestral *O. aragonica* in the Iberian Peninsula around 1.8 mya as reported by [47]. The node age was set to 1.8 ± 0.1 (SD) mya (1.96 to 1.64 mya, 95% HPD interval) (Additional file 3). (3) A combination of approaches 1 and 2, applying the dating of nodes mentioned above (Figure 5).

In the sites settings of BEAUti v.1.7.5 (part of the BEAST package [25]), the best fitting substitution models were applied separately to each of the three partitions and the node datings were assigned in the prior settings. The speciation model Yule Process [65] was chosen as tree prior. The BEAST analyses were each performed with four independent runs for 10^7 generations and every thousandth tree was sampled. Having checked whether the four runs had converged, these were combined with LogCombiner v.1.7.5 (part of the BEAST package). Subsequently, 25% of the trees were discarded as burnin and maximum clade credibility trees were calculated each from the remaining 30,000 trees.

The reconstruction of the geographic range history was performed in Lagrange [26], which uses a dispersal-extinction-cladogenesis (DEC) modeling for analyzing ML probabilities of rate transitions as a function of time. The rate-calibrated linearized tree from the molecular clock analysis (approach 3, two dating points) was prepared for Lagrange configurator [26], together with a range matrix in which each taxon/lineage was assigned to one of seven geographic regions, Northern Calcareous Alps (N), Southern Calcareous Alps (S), Western Alps (W), Western Carpathians (C), Dinarides (D), Western Romanian Carpathians (R) and Strandzha Mts. (Z). The maximum number of areas allowed for ancestors (= areas inhabited at the same time) was set to '2'. We tested two different models: (1) High migration probabilities ('1.0') were assigned to migration between directly adjacent areas, and lower probabilities ('0.5') were assigned to migration between not immediately adjacent areas (W to C; N to D; N to R). Migration was prohibited between geographically very distant areas (W to D; W to R; Z to W, N, S and C) (Figure 5). (2) Migration was permitted between all seven geographic regions with the same dispersal probabilities ('1.0') (Additional file 4).

The sequence alignments used for the calculation of the molecular clock tree are provided in Additional file 10.

Morphological analyses

A total of 526 specimens were analyzed in the morphometric analyses. The sample included only specimens of the group of Alpine endemics: *O. austriaca*

(66 specimens/12 localities), *O. a. faueri* Klemm, 1967 (60/4), *O. a. pseudofuchsi* Klemm, 1967 (21/2), *O. conica* (22/4), *O. fuchsi* (16/3), *O. gularis* (143/19), *O. pseudodolium* (141/14), *O. restituta* (18/2), *O. spoliata* (5/1) and *O. tolminensis* (34/4). Photographs of the shells were taken in frontal position with a WILD MAKROSKOP M420 and a NIKON DS Camera Control Unit DS-L2, all with the same magnification. Using tpsUtil v.1.44 [66], tps-files were created, then landmarks were set on 17 uniquely defined points of the shells' projections in tpsDig v.2.12 [67]. Landmarks 1, 2 and 9 (Figure 7D) are type I, landmarks 16 and 17 type III, and all other points are type II landmarks as defined in [68]. Qualitative characters such as the expression of the aperture folds are not considered in the morphometric analyses. Since the shell forms differ quite among specimens of the same sample localities and, thus, increase the variability of the data set, consensus shapes (here: mean shapes of several specimens of a single locality) were created with tpsSuper v.1.14 [69]. Linear discriminant analyses (LDA) were performed with the landmark data of all Alpine endemics. Plots of the mean shapes were calculated with R v.3.2.0 [70] by using RStudio v.0.97.551 [71]. Morphometric analyses of *O. dolium* are not performed here, this topic will be discussed in detail in a forthcoming publication (Harl et al. in prep).

Availability of supporting data

The data sets supporting the results of this article are included within the article and its supplementary files.

Additional files

Additional file 1: BI tree of the concatenated mitochondrial sequences (*12S, 16S* and *COI* 1st2nd positions). Posterior probabilities and ML bootstrap values are provided for all nodes above species level. The scale bar indicates the expected number of substitutions per site according to the models of sequence evolution applied. The black dots indicate nodes with high BI posterior probabilities (1.0) and ML bootstrap values (≥95). The colors of the species clades/labels correspond to those used in Figures 4, 7 and 9.

Additional file 2: Linearized molecular clock dated tree (approach 1). Maximum-clade–credibility tree calculated with BEAST, using the concatenated alignments of *12S, 16S* and *COI*. The root of the tree (node XIV) was calibrated to the age of the fossil *Nordsieckula falkneri*, the presumed most recent common ancestor of *Orcula*, *Sphyradium* and *Orculella*. Node bars indicate the 95% HPD ranges estimated for each node. Mean node ages and 95% HPD ranges are provided for the major splits. A time scale in mya is given below the tree. Abbreviations of the geological epochs: C: Chattian, A: Aquitanian, B: Burdigalian, L: Langhian, S: Serravallian, T: Tortonian, M: Messinian, Z: Zanclean, P: Piacenzian, G: Gelasian and C: Calabrian.

Additional file 3: Linearized molecular clock dated tree (approach 1). Maximum-clade–credibility tree calculated with BEAST, using the concatenated alignments of *12S, 16S* and *COI*. The divergence date of the outgroups *O. bulgarica* and *O. aragonica* (node XVI) was calibrated to the time of the first occurrence of ancestral *O. aragonica* in the fossil record. Node bars indicate the 95% HPD ranges estimated for each node. Mean node ages and 95% HPD ranges are provided for the major splits. A time

scale in mya is given below the tree. Abbreviations of the geological epochs: C: Chattian, A: Aquitanian, B: Burdigalian, L: Langhian, S: Serravallian, T: Tortonian, M: Messinian, Z: Zanclean, P: Piacenzian, G: Gelasian and C: Calabrian.

Additional file 4: Reconstruction of the historic geographic ranges (unconstrained model). The linearized molecular clock dated maximum-clade-credibility tree shows the relationships of selected mt lineages (concatenated *16S, 12S* and *COI sequences*). Migration was permitted between all areas and with the same dispersal probabilities. Black dots indicate nodes with high posterior probabilities. The colored symbols at the branch tips indicate the geographic origin of each haplotype. At the cladogenesis events (nodes), all alternative ancestral subdivision/inheritance scenarios with likelihoods of 10% or more are indicated, separated by an "or", together with the respective likelihoods in%. When scenarios for cladogenesis events involve two ancestral areas, the symbol for the likely ancestral area/-s is/are provided left to each of the two branches. For nodes representing major splits, node ages and 95% posterior HPD intervals are indicated (see Table). A time scale in mya is given below the tree. Abbreviations of the geological epochs: C: Chattian, A: Aquitanian, B: Burdigalian, L: Langhian, S: Serravallian, T: Tortonian, M: Messinian, Z: Zanclean, P: Piacenzian, G: Gelasian and C: Calabrian.

Additional file 5: Mean and maximum genetic *p*-distances in the *12S* data set.

Additional file 6: Mean and maximum genetic *p*-distances in the *16S* data set.

Additional file 7: Mean and maximum genetic *p*-distances in the *H4/H3* data set.

Additional file 8: Sequence alignments (*COI, 12S, 16S*) used for the reconstruction of the combined mt tree (Figure 3).

Additional file 9: Sequence alignment used for the reconstruction of the nc *H4/H3* tree (Figure 4).

Additional file 10: Sequence alignments (*COI, 12S, 16S*) used for the reconstruction of the molecular clock tree (Figure 5).

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In Search of Glacial Refuges of the Land Snail *Orcula dolium* (Pulmonata, Orculidae) - An Integrative Approach Using DNA Sequence and Fossil Data

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Abstract

Harboring a large number of endemic species, the Alps and the Western Carpathians are considered as major centers of biodiversity. Nonetheless, the general opinion until the turn of the millennium was that both Central European mountain regions did not provide suitable habitat during the Last Glacial Maximum, but were colonized later from southern refuges. However, recent molecular genetic studies provide new evidence for peripheral Alpine refuges. We studied the phylogeography of the calciphilous land snail *O. dolium* across its distribution in the Alps and the Western Carpathians to assess the amount of intraspecific differentiation and to detect potential glacial refuges. A partial sequence of the mitochondrial COI was analyzed in 373 specimens from 135 sampling sites, and for a subset of individuals, partial sequences of the mitochondrial 16S and the nuclear histone H3 and H4 were sequenced. A molecular clock analysis was combined with a reconstruction of the species' geographic range history to estimate how its lineages spread in the course of time. In order to obtain further information on the species' past distribution, we also screened its extensive Pleistocene fossil record. The reconstruction of geographic range history suggests that *O. dolium* is of Western Carpathian origin and diversified already around the Miocene-Pliocene boundary. The fossil record supports the species' presence at more than 40 sites during the last glacial and earlier cold periods, most of them in the Western Carpathians and the Pannonian Basin. The populations of *O. dolium* display a high genetic diversity with maximum intraspecific *p*-distances of 18.4% (COI) and 14.4% (16S). The existence of various diverged clades suggests the survival in several geographically separated refuges. Moreover, the sequence patterns provide evidence of multiple migrations between the Alps and the Western Carpathians. The results indicate that the Southern Calcareous Alps were probably colonized only during the Holocene.

Introduction

The Pleistocene climate changes shaped the phylogeographic patterns of various organisms [1]. In particular, the severe cooling starting with the end of the Early Pleistocene (about 900 kya) was the starting point for massive glaciations in the northern hemisphere [2]. Mountainous regions such as the Central European Alps were heavily affected due to shifts in temperature and humidity, and the expansion of glaciers, resulting for many taxa in the fragmentation of populations, complete or local extinction, and the loss of variation due to genetic bottlenecks [1]. Thereby, the Last Glacial Maximum (LGM; 30–18 kya [3]) is of most relevance in respect to the current distribution of Central European species. Although the existence of glacial refuges at the periphery of the Alps was discussed more than half a century ago [4], the general opinion until end of the 20th century was that glacial refuges were located mainly in southern regions [1]. However, molecular genetic analyses and fossil data revealed the existence of northern refuges in the Western Carpathians [5,6] and the Pannonian Basin [7]. Several peripheral Alpine refuges were proposed for silicophilous mountain plants [8] and

calciphilous land snails such as *Arianta arbustorum* [9–12], *Carychium minimum*, *Carychium tridentatum* [13], *Trochulus oreinos* [14] and *Trochulus villosus* [15].

Most molecular genetic studies investigating glacial refuges were based on the assumption that populations diverged during isolation in geographically separated areas, and that populations of former refuge areas are now characterized by high genetic diversity and the presence of rare (private) alleles [16]. Nevertheless, a major obstacle in identifying signatures of Pleistocene refuges is that phylogenetic signals are blurred because of migration and intermixture of previously separated populations. Therefore, species showing low dispersal capabilities and specific habitat requirements, which is the case in most land snails, might be suited to infer past distributional patterns.

In the present study, we investigate the phylogeographic patterns of the land snail *Orcula dolium* (Draparnaud, 1801). The species inhabits all major limestone areas of the Alps and the neighboring Western Carpathians [17,18]. *O. dolium* is usually associated with mountainous forest habitats or rocky landscapes with patches of vegetation, but it is also found on rocky slopes at high altitudes up to 2160 m above sea level (asl) [17] (and data of

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present study). Within the Alps, the east-west orientated Central Alps, consisting mainly of silicate rock, represent the largest distributional barrier for the species; they separate the Northern Calcareous Alps from the Southern Calcareous Alps. The Vienna Basin, closing the Pannonian Basin to the north, constitutes another distributional gap because it separates the populations of the Northern Calcareous Alps (including the Wienerwald) and the Western Carpathians. Recent investigations of loess profiles from the Pannonian Basin show that during the Late Pleistocene *O. dolium* also occurred in the lowlands of the region [19], although the species seems to have vanished from it during the Holocene. The margins of the Northern Calcareous Alps and the Western Carpathians, enclosing the Vienna Basin east and west, harbor the majority of the described 23 subspecies [20]. As these areas were only partially glaciated during the LGM, they also come into consideration as potential refuges for the species.

We perform a comprehensive phylogeographic study of *O. dolium* analyzing mt and nc markers to detect potential glacial refuges and to assess the amount of intraspecific variability. Furthermore, we test whether the described subspecies are differentiated genetically in the mitochondrial (mt) and nuclear (nc) markers. Our sample covers almost the entire range of the species. We include data of the Pleistocene fossil record to obtain insights into the species' past distribution. In order to estimate which potential areas were inhabited by ancestral populations of *O. dolium* and to trace the distribution patterns of the mt lineages throughout time, a molecular clock analysis is performed and combined with a phylogeographic range reconstruction.

Methods

Study Area and Sampling

Specimens were collected from a large part of the species' distribution, including several Alpine and Western Carpathian type localities. *O. dolium* is not protected by conservation laws of the countries where the collections were performed. Thus, in general, permissions were not necessary. For protected areas in Austria, permissions were provided by federal states authorities. Permit numbers: RU5-BE-64/011-2013 (Lower Austria), FA13C-53 Sch 6/6–2007 (Styria) and N10-117-2008 (Upper Austria). Most samples of *O. dolium* were collected in the Northern Calcareous Alps (Austria and Germany), a lesser fraction in the Western Carpathians (Slovakia), the Southern Calcareous Alps (Austria, Slovenia and Italy) and the Western Alps (Switzerland), totaling 373 specimens of 135 sites (Table 1). The habitats include wooded areas in the lowland, mountainous vegetated areas and rocky slopes in the alpine zones, with an altitudinal range from 120 m to 2160 m asl. Elevation and position were determined via GPS. At every sample locality, if available, a minimum of three living specimens was collected, prepared for DNA analyses and stored in 80% ethanol following the protocol of [21]. *Orcula conica* (Rossmässler, 1837) (ID: 3899; Trögener Klamm, Carinthia) was used as outgroup. Selected type specimens of *O. dolium* subspecies included in the present study are shown in Fig. S1. For the inference of substitution rates used in the molecular clock analysis, six specimens of *Orculella bulgarica* (Hesse, 1915) and four specimens of *Orculella aragonica* (Westerlund, 1897) were included. Samples of *Orculella bulgarica* were collected by Barna Páll-Gergely (Shinshu University, Matsumoto, Japan; Turkish samples) and Alexander Reischuetz (Greek samples). DNA samples of *O. aragonica* were obtained from Benjamín Gómez-Moliner (Universidad del País Vasco, Vitoria, Spain). Voucher specimens of the first three taxa are deposited in the Natural History Museum Vienna, the whereabouts of the *O. aragonica* vouchers are provided in [22].

Every individual sample was assigned an ID consisting of a unique specimen number and a locality tag. The latter encodes the Alpine geographic mountain region as classified in the SOIUSA system [23], with localities of each region numbered from west to east (Table 1). Due to their geographic vicinity to the Northern Calcareous Alps, the sites located in the Fischbach Alps, Eastern Styrian Prealps and Lavanttal Alps as well as those of the Wienerwald, which are classified to the Central Alps in the SOIUSA system, are treated as Northern Calcareous Alpine mountain regions here. A map providing an overview of the mountain regions investigated is shown in Fig. 1. The Slovakian sites were each assigned to one of the geological areas defined for the Carpathians [24]. To illustrate distribution patterns, the haplotypes in the phylogenetic trees and the histone network are marked by different colors, corresponding to the SOIUSA mountain regions as shown in Fig. 1.

Molecular Markers and Primer Design

DNA was extracted from 373 *O. dolium* specimens with the QIAgen Blood and Tissue Kit, using a piece of foot tissue. A partial region of the mt cytochrome c oxidase I gene (COI) was sequenced in all 373 specimens. From a subset of 54 individuals (including representatives of the major mt clades, type localities, peripheral geographic regions and contact zones of distinct mt clades), two additional markers were amplified: a section of the mt 16S gene, as well as a nc sequence comprising almost the entire sequences of the histone 4 and 3 genes and the complete internal spacer region (H4/H3). The two histone genes are orientated in opposite direction and are separated by a non-coding spacer region, an arrangement which is probably not universal in gastropods but was verified so far particularly for species of the informal group of Orthurethra (sensu [25]). The COI and 16S sequences were also amplified in the eleven outgroup specimens. The COI forward primer *COIfolmerFwd* [14] is a variant of the standard primer *LCO1490* [26]; *H2198-Alb* [10] was used as reverse primer. New 16S primers were designed for the amplification of a fragment of approximately 850 bp. The forward primers, *16SLOrc1_fw* and *16SLOrc2_fw*, bind about 50 bp away from the 5′-end of the *16S rRNA* gene and the reverse primer *16SLOrc_rv* is situated in a conserved region about 850 bp downstream. The H4/H3 primers, *OrcH4_left1* and *OrcH4_left2* (positioned at the 3′-end of the H4 gene) were designed based on alignments of H3 and H4 sequences from GenBank. The reverse primer *OrcH3_right1* (at the 3′-end of the H3 gene) was published by [27]. Internal primers for sequencing (*OrcH3S_right3* and *OrcH4S_left3*) were positioned close to the spacer in the coding sequences to obtain the complete 1100 bp fragment with two sequencing runs. The PCR primers (*OrcH4_left1*, *OrcH4_left2* and *OrcH3_right1*) cover a wider spectrum of Orthurethra taxa, while the internal primers (*OrcH3S_right3* and *OrcH4S_left3*) were especially adapted to the genus *Orcula*. All primers are listed in Table 2.

PCR Amplification and Cloning

COI and 16S fragments were amplified with the Roche Taq DNA polymerase for direct sequencing. The PCR started with a denaturation step for 3 min at 94°C, followed by 35 cycles with 30 s at 94°C, 30 s at the particular annealing temperature (Table 2), and 1 min at 72°C, followed by a final extension for 7 min at 72°C. The PCR for the nc H4/H3 fragments was performed with the standard protocol of the Finnzymes Phusion polymerase, which has proofreading activity. PCR started with a denaturation step for 30 s at 98°C, followed by 35 cycles with 10 s at 98°C, 10 s at the particular annealing temperature (see Table 2),

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Table 1. List of localities and individuals included in the present study.

locality code	Locality	N	E	m. asl	clade (mt)	histone alleles	IndID	Sub-species
Western Carpathians								
MLF1	SK, TC, Považská Bystrica, Považský hrad	49°8.734'	18°27.422'	500	2, 3, 9	HT1 var	3937 (2); 3936, 3938, 3940, 3941, 3943 (3); 3939, 3942 (9);	*d*
MLF2	SK, TC, Považská Bystrica, Manínska tiesňava	49°8.398'	18°30.421'	380	1	HT3	3918, 3919, 3920, 3921, 3922, 3923, 3924, 3925	***b*** *
MLF3	SK, TC, Považská Bystrica, Manínska tiesňava	49°8.366'	18°30.475'	400	1	HT3 var/HTX	3912, 3913, 3914, 3915, 3917	***b*** *
MLF4	SK, ZI, Súľov-Hradná, Súľovské skaly	49°10.101'	18°34.633'	315	1, 9	HT3	3930, 3931, 3932, 3934, 3935 (1); 3933 (9)	*d*
MLF5	SK, ZI, Malá Fatra, Fackov	49°0.007'	18°35.853'	556	7, 10	HTX	1374 (7); 1375, 1376, 1380 (10);	*d*
MLF6	SK, ZI, Rajecké Teplice, Skalky Strážovské	49°8.115'	18°41.745'	470	1, 3	HT3/HTX	3952, 3953, 3954 (1); 3944, 3945, 3946, 3947, 3948, 3949, 3950, 3951 (3);	***m*** *
MLF7	SK, ZI, Malá Fatra, Terchová-Vrata	49°14.664'	19°2.36'	564	7	HTX	1372, 1373	*d*
STR1	SK, TC, Trenčianske Teplice, Malý Klepáč	48°53.72'	18°10.649'	480	9	HT1	3908, 3909, 3910	***t*** *
STR2	SK, TC, Strážovské vrchy, Valaska Beta	48°53.519'	18°22.469'	445	9	HTX	1996	*d*
VEF1	SK, ZI, Ružomberok, Cebrat (S side)	49°5.474'	19°17.174'	700	5	HT3	3926, 3927, 3928, 3929	***c***
Northern Calcareous Alps								
BGD1	DE, BY, Ruhpolding, Brand	47°45.004'	12°37.92'	681	1		4088, 4089, 4090	*d*
BGD2	DE, BY, Bad Reichenhall, Obernesselgraben	47°42.593'	12°48.569'	605	1		4086, 4087	*d*
BGD3	AT, S, Steinernes Meer, Oberweissbachgraben (mid)	47°28.288'	12°51.716'	740	8		4113, 4114, 4115	*d*
BGD4	AT, S, Steinernes Meer, Einsiedler	47°26.622'	12°51.72'	1029	8	HT2	4117, 4118, 4119	*d/e/r*
BGD5	AT, S, Steinernes Meer, Oberweissbachgraben (E end)	47°28.298'	12°51.997'	850	8		4109, 4110, 4111	*d/e/r*
BGD6	AT, S, Steinernes Meer, Wiechenthalerhütte (ascent)	47°27.652'	12°52.122'	1413	8		4121	*d/e/r*

Table 1. Cont.

locality code	Locality	N	E	m. asl	clade (mt)	histone alleles	IndID	Sub-species
BGD7	DE, BY, Wimbachtal, Mittleres Wimbachtal	47°34.596'	12°54.064'	1000	1		4083, 4084, 4085	*d/e/r*
BGD8	AT, S, Untersberg, Fürstenbrunn	47°44.523'	12°59.582'	515	1		4126, 4127, 4128	*d*
BGD9	AT, S, Untersberg, Untersberg (N side)	47°43.703'	13°0.497'	1530	1		4122, 4123	*d/e/r*
BGD10	DE, BY, Untersberg, Eckberg	47°41.312'	13°1.089'	747	1		4079, 4080, 4081	*d*
BGD11	AT, S, Hochkönig, Dientner Sattel (Elmau)	47°23.713'	13°4.924'	1256	1, 8	HT1 var	1173 (1); 1172 (8);	*d*
DAC1	AT, OOE, Dachstein, Klausbrunn	47°32.787'	13°36.755'	940	1		1357, 1377, 1378	*d*
DAC2	AT, S, Salzberg, Hallstatt (Plassenwanderweg 1177)	47°34.151'	13°37.482'	1177	1		1125	*d*
DAC3	AT, OOE, Dachstein, Wiesbergalm (Wiesberghaus)	47°31.529'	13°37.493'	1685	8	HT1 var	1279, 1280, 1281	*d*
DAC4	AT, ST, Grimming, Grimming (SE ascent 2)	47°31.172'	14°2.326'	1149	8	HT2	5641, 5642, 5643	*d*
DAC5	AT, ST, Grimming, Grimminghütte	47°30.865'	14°2.962'	938	8		5638, 5639, 5640	*d*
ENN1	AT, ST, Haller Mauern, Großer Pyhrgas (Hiaslalm)	47°38.638'	14°22.595'	1292	1		3083, 3084, 3085	*d*
ENN2	AT, ST, Johnsbachtal, Langriesgraben	47°33.641'	14°34.64'	704	1		1135, 1136, 1137	*d*
ENN3	AT, ST, Johnsbachtal, Langries bridge	47°33.681'	14°34.845'	631	1		5646, 5647, 5648	*d*
ENN4	AT, ST, Johnsbachtal, Kaderalpl	47°34.063'	14°34.899'	634	1		874, 875, 876	*d*
ENN6	AT, ST, Johnsbachtal, Im Gseng 1 (below)	47°34.044'	14°34.934'	605	1		5654	*d*
ENN8	AT, ST, Johnsbachtal, Hellichter Stein	47°34.544'	14°35.348'	606	1		913, 914, 916, 918	*d*
ENN9	AT, ST, Johnsbachtal, Im Gseng 3 (mid part)	47°34.085'	14°35.687'	766	1		5618	*d*
ENN10	AT, ST, Johnsbachtal, Im Gseng 4	47°34.033'	14°36.106'	1039	1		5623, 5624, 5625	*d/e/r*
ENN11	AT, ST, Haindlkar, Zigeuner	47°35.114'	14°36.695'	603	1		893, 894, 895	*d*
ENN12	AT, ST, Großes Haindlkar, Haindlkar-Hütte 2	47°34.039'	14°36.773'	1078	1		5632, 5633, 5634	*d*

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Table 1. Cont.

locality code	Locality	N	E	m. asl	clade (mt)	histone alleles	IndID	Sub-species
ENN13	AT, ST, Gstatterboden, Ameishütte	47°35.744'	14°38.25'	629	1		1128, 1129	*d*
ENN14	AT, ST, Gstatterboden, Ameishütte	47°35.744'	14°38.25'	629	1		841, 842	*d*
ENN15	AT, ST, Gstatterboden, Planspitzgraben	47°35.297'	14°38.327'	668	1		897, 910, 911	*d*
ENN16	AT, ST, Johnsbachtal, Schröckalm	47°32.072'	14°40.051'	1377	1		1166, 1167, 1168	*d/e/r*
ENN17	AT, ST, Tamischbachturm, ridge on summit	47°36.932'	14°41.76'	1940	1		3887, 3889, 3890	*d/e/r*
ENN18	AT, ST, Großreifling, Großreifling	47°39.481'	14°42.669'	450	1		1158, 1159, 1160	*d*
ENN19	AT, ST, Hieflau, Schneckensafari	47°36.013'	14°44.778'	523	1		3106, 3107, 3108	*d*
FOB1	AT, ST, Schöckl, Teufelstein	47°12.525'	15°27.665'	950	1	HT1	858, 859, 860	*p*
FOB2	AT, NOE, Semmering, Roter Berg (Kalte Rinne)	47°39.411'	15°47.694'	822	1		629, 630, 837	*d/g/p*
FOB3	AT, NOE, Semmering, Sonnwendstein (Almweg 1229)	47°37.604'	15°51.141'	1229	1	HT1/HT1 var	370, 371, 372	*d/g/p/e/r*
FOB4	AT, NOE, Semmering, Sonnwendstein (Almweg 1343)	47°37.682'	15°51.228'	1343	1		606, 1121, 1122	*d/g/p/e/r*
FOB5	AT, NOE, Semmering, Sonnwendstein summit	47°37.783'	15°51.62'	1523	1		339	*d/g/p/e/r*
FOB6	AT, NOE, Semmering, Sonnwendstein (Pollereshütte)	47°37.751'	15°51.708'	1477	1		361, 367	*d/g/p/e/r*
FOB8	AT, NOE, Semmering, Mariaschutz (Marterl)	47°38.276'	15°52.63'	788	1		602, 603, 604, 1984	*d/g/p*
GUT1	AT, NOE, Tiefental, Ochbauer	47°52.638'	15°38.854'	739	6	HTX	3041, 3042, 3043	*d*
GUT2	AT, NOE, Halbachtal, Rossbachklamm 1	47°54.327'	15°40.937'	649	6		3074, 3075, 3076	*d*
GUT3	AT, NOE, Halbachtal, Kleinzell	47°56.902'	15°42.874'	507	6		3066, 3067, 3068	*d*

Table 1. Cont.

locality code	Locality	N	E	m. asl	clade (mt)	histone alleles	IndID	Sub-species
GUT4	AT, NOE, Gösing, Sieding (foothill Gösing)	47°44.399'	15°58.723'	356	1		5471	*p*
GUT5	AT, NOE, Gösing, Gösing (foothill W side)	47°44.431'	15°59.198'	700	1		5472, 5473, 5474, 5475	*p*
GUT6	AT, NOE, Gösing, Gösing (W side)	47°44.401'	15°59.205'	864	1		2942, 2943, 2945	*d/p/e/r*
GUT7	AT, NOE, Gösing, Flatzerwand (Gösing)	47°44.829'	16°0.215'	671	1		2934, 2935, 2936	*p*
GUT8	AT, NOE, Hohe Wand, Grafenberg (Seiser Toni)	47°48.360'	16°0.503'	780	6	HT1	298, 299, 1134	*d*
HSC1	AT, ST, Hochschwab, Aflenzer Staritzen	47°38.279'	15°16.095'	1779	1		1358, 1392	*d/e/r*
HSC2	AT, ST, Hochschwab, Salzatal (Weichselboden)	47°39.96'	15°9.812'	660	1		3078, 3079, 3081	*d/e/r*
KAI1	AT, T, Wilder Kaiser, Kaiserkopf	47°33.225'	12°18.223'	1549	1		1138, 1140, 1982	*d*
KAI2	AT, T, Wilder Kaiser, Rote Rinne	47°33.624'	12°18.238'	2159	1		1119, 1120	*d*
KAI3	AT, T, Wilder Kaiser, Gamsanger	47°33.682'	12°18.428'	1938	1		838, 1107, 6506	*d*
KAI4	AT, T, Wilder Kaiser, Hochgrubach	47°33.357'	12°18.604'	1666	1	HT1	1142, 1143, 1981	*d*
KWL1	AT, T, Pertisau, Achensee W shore	47°27.423'	11°42.081'	955	1	HT1	614, 615, 617, 1983	*d*
LAV1	AT, ST, Gleinalpe, St. Pankrazen (Stübinggraben)	47°9.466'	15°10.934'	730	1		864, 865, 866	*p*
LEC1	AT, T, Imst, Hahntennjoch	47°17.225'	10°36.566'	1482	1	HT1	5932	*d*
MRZ1	AT, NOE, Göller, Gscheid	47°48.622'	15°27.084'	914	1		2976, 2977, 2978	*d/e/r*
MRZ2	AT, NOE, Göller, Lahnsattel	47°46.493'	15°29.145'	1020	1		2972, 2973, 2974	*d/e/r*
MRZ3	AT, NOE, Göller, Turmmauer	47°48.705'	15°31.118'	812	1		3028, 3030	*d/e/r*
MRZ4	AT, NOE, Göller, Klopfermauer	47°48.705'	15°32.264'	741	1		2961, 2962, 2963, 2964	*d*
MRZ5	AT, NOE, Göller, Klopfermauer waterfall	47°48.705'	15°32.264'	741	1		2970, 2971	*d*

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Table 1. Cont.

locality code	Locality	N	E	m. asl	clade (mt)	histone alleles	IndID	Sub-species
MRZ6	AT, ST, Hohe Veitsch, Wildkamm 2	47°39,676'	15°23,437'	1421	1		6149, 6150	*d/e/r*
MRZ7	AT, ST, Hohe Veitsch, Wildkamm 1	47°39,396'	15°24,264'	1587	1		6146, 6147	*d/e/r*
OOV2	AT, OOE, Traunstein, Gmundner-Hütte	47°52,323'	13°50.016'	1604	8	HT2	599, 600, 601	*d/e/r*
OOV3	AT, OOE, Traunstein, Mairalmsteig	47°51.96'	13°50.047'	1166	8		2810, 2811	*d/e/r*
OSR1	AT, NOE, Grimmenstein, Burgruine Grimmenstein	47°37.949'	16°7.289'	677	1	HT1 var	5611, 5613	*p*
OSR3	AT, NOE, Seebenstein, Türkensturz	47°40.876'	16°8.277'	550	1	HT1	5606, 5607, 5608	*p* *
ROF1	AT, T, Hochiss, Mauritz-Hochleger	47°26.629'	11°45,91'	1832	1		620, 621, 622	*d*
SNH11	AT, ST, Schneealpe, Kampl (Kutatschhütte)	47°41.096'	15°36.182'	1673	1		3328, 3329, 3330	*d/e/r*
SNH12	AT, ST, Schneealpe, Kampl (Kohlebnerstand)	47°40.871'	15°36.677'	1487	1		3324, 3325, 3327	*d/e/r*
SNH13	AT, ST, Schneealpe, Schauerkogel (Schneealpenhaus)	47°41.875'	15°36.696'	1742	1		3332, 3333, 3334	*d/e/r*
SNH14	AT, NOE, Rax, Preiner Gscheid (Waldbach 1)	47°39.395'	15°41.097'	817	1		2947, 2948	*d/e/r* *
SNH15	AT, NOE, Rax, Preiner Gscheid (Waldbach 2)	47°39.395'	15°41.097'	817	1		2950	*d/e/r* *
SNH16	AT, NOE, Rax, Preiner Gscheid	47°40.981'	15°43.124'	1249	1		1123	*d/e/r* *
SNH17	AT, NOE, Rax, Seehütte	47°42.173'	15°43.33'	1761	1	HT1 var	315, 316, 317	*d/e/r* *
SNH18	AT, NOE, Rax, Göbl-Kühnsteig 1	47°41.42'	15°43.399'	1397	1		1111	*d/e/r* *
SNH19	AT, NOE, Höllental, Weichtalklamm	47°44.883'	15°46.249'	592	1		349, 350, 351	*d*
SNH20	AT, NOE, Rax, Gsolhirn-Steig	47°43.141'	15°46.993'	1400	1		3859, 3860, 3862	*d/e/r* *
SNH21	AT, NOE, Schneeberg, Fadenwände 1525	47°47.253'	15°48.655'	1525	6	HT1 var	1362	*e/r* *
SNH22	AT, NOE, Schneeberg, Schneidergraben	47°46.096'	15°49,99'	1401	1	HT1 var	1387, 1389	*e/r* *
SNH23	AT, NOE, Semmering, Adlitzgraben (Breitenstein)	47°39.361'	15°50.168'	650	1		395, 396, 397, 2814, 2815	*g/p* *

Table 1. Cont.

locality code	Locality	N	E	m. asl	clade (mt)	histone alleles	IndID	Sub-species
SZK1	AT, S, Vordertrattbach, Lungauer Kurve	47°38.284'	13°15.425'	1295	1		1506, 1507	*d*
SZK2	AT, S, Seewaldtal, Seewaldsee	47°37.757'	13°16.244'	1115	1	HT1 var	1518, 1519, 1520	*d*
SZK3	AT, S, Trattberg, Christlalm	47°39'	13°16.7'	1441	1		1508	*d*
SZK4	AT, S, Trattberg, Parkplatz	47°38.352'	13°16.821'	1551	1		1501, 1502, 1503	*d*
SZK5	AT, OOE, Hochlecken, Aurach Ursprung	47°50.308'	13°37.411'	857	8		288, 292	*d/e/r*
SZK6	AT, OOE, Höllenkogel, Riederhütte	47°48.238'	13°40.602'	1757	8		636, 637, 831	*d*
SZK7	AT, OOE, Höllenkogel, Totengrabengupf	47°48.272'	13°41.192'	1736	8		607, 609	*d/e/r*
SZK8	AT, OOE, Hochschneid, Hochschneid	47°48.458'	13°41.533'	1624	8	HT1	346, 347, 348	*d*
SZK9	AT, OOE, Steinkogel, Großer Steinkogel	47°48.737'	13°42.732'	1531	8		648, 649, 650	*d*
SZK10	AT, OOE, Feuerkogel, Pledialm	47°48.984'	13°43.549'	1444	8		644, 1145, 1146	*d/e/r*
TEN1	AT, S, Hochthron, Unteres Throntal	47°29.461'	13°14.091'	1717	8	HT1 var	1488, 1489, 1490	*d/e/r*
TEN2	AT, S, Hochthron, Thronleiter	47°29.31'	13°14.611'	1940	8	HT1 var	1523, 1524	*d/e/r*
TEN3	AT, S, Hochthron, Werfenerhütte	47°29.311'	13°14.611'	1969	1, 8	HT1 var	1495 (1); 1493, 1494 (8);	*d/e/r*
TOT1	AT, OOE, Großer Priel, Vorderer Ackergraben	47°43.724'	14°2.504'	1027	8		3863, 3864, 3865	*d*
TOT2	AT, OOE, Großer Priel, Mittlerer Ackergraben	47°43.48'	14°2.69'	1495	8		3879, 3880, 3881	*d*
TOT3	AT, OOE, Großer Priel, Welser-Hütte	47°43.493'	14°2.99'	1747	8	HT2	3871, 3872, 3873	*d*
WIW2	AT, NOE, St. Andrä-Wördern, Hagenbachklamm	48°18.66'	16°12.582'	191	4B	HT1 var/ HT2 var/ HTX	385, 386, 387, 392, 393, 394	*i*
WIW3	AT, W, Mauerbach, Mauerbachtal	48°13.743'	16°12.796'	205	4B	HT2	1161, 1162	*i*
WIW8	AT, NOE, Greifenstein, Kaiserdenkmal	48°20.615'	16°15.205'	253	4B	HT2	628	*i*
YBB1	AT, NOE, Dürrenstein, Herdenglhöhle (Herdengl)	47°50.674'	14°59.006'	850	1		1148, 1149	*d/e/r*

Table 1. Cont.

locality code	Locality	N	E	m. asl	clade (mt)	histone alleles	IndID	Sub-species
YBB2	AT, NOE, Dürrenstein, Lechnergraben (Wildgatter)	47°49,94'	15°1,589'	674	1		1163, 1164, 1165	*d*
YBB3	AT, NOE, Dürrenstein, Wiesenalm (Hühnerkogel)	47°48,862'	15°2,034'	1475	1		1126, 1127	*e/r*
YBB4	AT, NOE, Dürrenstein, Am Hohen Hirzeck (Hühnerkogel)	47°48,987'	15°2,11'	1410	1		313, 834, 835	*e/r*
YBB5	AT, NOE, Dürrenstein, Rosseck (Legsteinquelle)	47°48,045'	15°2,523'	1480	1		96, 97, 98, 306, 308	*e/r*
YBB6	AT, NOE, Dürrenstein, Springkogel (Dolinenrand)	47°47,561'	15°2,803'	1665	1		92, 93, 94	*e/r*
YBB7	AT, NOE, Dürrenstein, Lechnergraben (Talschluss)	47°49,387'	15°2,673'	1160	1		300	*e/r*
YBB8	AT, NOE, Dürrenstein, Dürrenstein (ascent E side)	47°47,494'	15°4,102'	1631	1		95, 1124	*e/r*
YBB9	AT, ST, Kräuterin, Dürradmer	47°43,231'	15°9,64'	1100	1		1152	*d*
YBB10	AT, NOE, Ötscher, Hüttenkogel	47°51,297'	15°11,171'	1605	1		3883, 3884	*d/e/r*
YBB11	AT, NOE, Hochbärneck, Reifgräben	47°56,98'	15°11,83'	420	1		1933	*d*
YBB12	AT, NOE, Hochbärneck, Hochbärneck	47°55,047'	15°12,032'	850	1		1934, 1935, 1936	*d/e/r*
Southern Calcareous Alps								
GAI1	AT, K, Kreuzen, Meierle	46°41,028'	13°26,015'	927	1	HT1	1131, 1132, 1133	*d*
GAI2	AT, K, Kreuzen, Gailwaldbachgraben	46°40'	13°34,006'	880	6		1108, 1109	*d*
GAI3	AT, K, Kreuzen, Gailwaldbachgraben	46°40,149'	13°34,28'	880	6		1150, 1151	*d*
GAI4	AT, K, Dobratsch, Höhenrain	46°35,048'	13°41,041'	1900	6	HT1	640, 642	*d/e/r*
JUL1	IT, Bovec, Mt. Predil, Predil pass	46°25,148'	13°34,59'	1153	6		3461	*d*
JUL2	SI, Bovec, Koritnica valley, Trdnjava Kluže 2	46°21,61'	13°35,62'	534	6		1944, 1945	*d*
JUL3	SI, Bovec, Koritnica valley, Trdnjava Kluže 1	46°21,61'	13°35,62'	534	6		3465	*d*
JUL4	SI, Bovec, Mt. Triglav, Kluže	46°20,8'	13°30,9'	870	6	HT1	1363, 1364, 1365	*d*

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Table 1. Cont.

locality code	Locality	N	E	m. asl	clade (mt)	histone alleles	IndID	Sub-species
JUL5	IT, FVG, Fusine in Valromana, Mangart	46°27.496'	13°41.12'	1379	6		1509, 1510, 1511	d
KWN1	AT, K, Koschuta, Trögener Klamm	46°27.212'	14°29.811'	762	6	HT1	3903, 3905, 3906	d
Western Alps								
GLA1	CH, SG, Calfeisental, St. Martin	46°55.353'	9°21.333'	1347	4A	HT1/HT1 var	5933, 5934, 6140	d
SML11	CH, BRN, Rumisberg, Schore (N of)	47°16,671'	7°38,210'	1066	4		6140	d
SML4	CH, BRN, Moutier, Gorges de Court	47°15.36'	7°20.61'	650	4A	HT1	6144, 6145	d
Outgroup taxa								
Orcula conica								
KWN1	A, K, Koschuta, Trögener Klamm	46°27.212'	14°29.811'	762			3899	
Orculella aragonica								
BAE1	ES, Andalucia, Granada, Graena	31°18.44'	−3°14.81'	940			7132 (P3)	
BAE2	ES, Andalucia, Granada, Tocón	31°12,47'	−3°21.63'	1431			7133 (P6)	
BAE3	ES, Andalucia, Granada, El Baico	37°31.95'	−2°44.38'	1431			7135 (P7)	
BAE4	ES, Andalucia, Granada, Tocón	37°15.16'	−3°23,67'	1365			7137 (P9)	
Orculella bulgarica								
PIN1	GR, West Macedonia, Florina	40°44.1'	21°40.7'	580			7002, 7003, 7004	
PON1	TR, Erzurum, Aşkale	39°50,4'	40°33.9'	1880			6606, 6607	
PON2	TR, Erzurum, Aşkale	39°56.5'	40°36.4'	1652			7105	

The first column indicates the mountain chain: Northern Calcareous Alps (NCA), Southern Calcareous Alps (SCA), Western Alps (WA) and Western Carpathians (CAR). The SOIUSA codes each correspond to a mountain region following [23] (see Fig. 1 for full names). Country names are abbreviated according to the ISO 3166-1 code as defined by the International Organization for Standardization. The GPS coordinates are given according to the World Geodetic System 1984 (WGS84) alongside the altitude in meters above sea level (asl). The individual IDs (IndIDs) of the specimens, together with information on the respective mt clades, are provided for each locality. The attachment 'var' indicates that specimens provided variants slightly deviating from the three main alleles, and 'HTX' are strongly differing unique alleles. The last column indicates the subspecies reported by [17] for the respective Alpine areas (e: edita, r: raxae, p: pseudogularis, i: infima, d: dolium, g: gracilior) or represent type localities of Carpathian subspecies (b: brancsikii, c: cebratica, m: minima, t: titan). Asterisks indicate that the sampling site is the type locality of the respective subspecies. Abbreviations of country names and federal districts are provided at the end of the table.
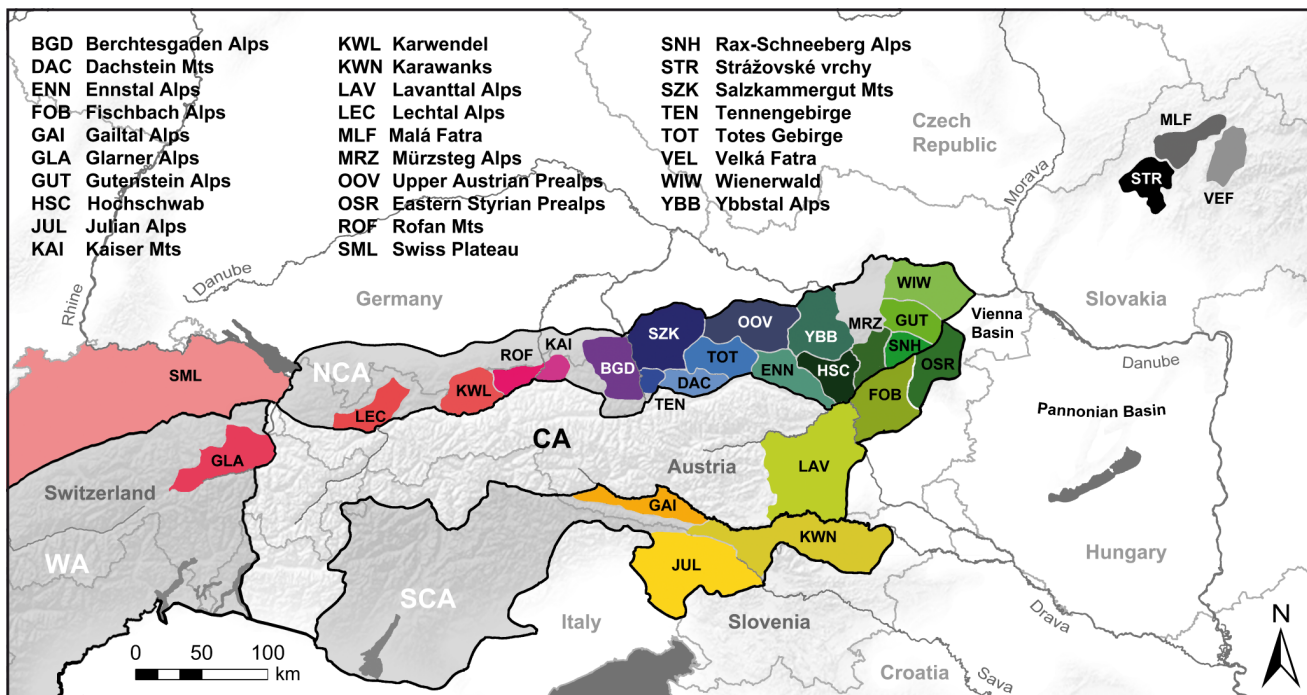
doi:10.1371/journal.pone.0096012.t001

Figure 1. Distribution of mountain areas investigated in the present study. The colors correspond to those used in the mt trees (Figs. 1 and 4) and in the histone H4/H3 network (Fig. 5). The outlines of the Northern Calcareous Alps (NCA), the Southern Calcareous Alps (SCA), the Western Alps (WA) and the Central Alps (CA) are framed in black. The names of the mountain regions and abbreviations are provided in the figure.
doi:10.1371/journal.pone.0096012.g001

and 30 s at 72°C, followed by a final extension for 7 min at 72°C. Purification and sequencing of the PCR products (both directions) were performed at LGC Genomics (Berlin, Germany), using the PCR primers for sequencing, except for the H4/H3 section, which was sequenced with the internal primers *OrcH3S_right3* and *OrcH4S_left3*. The cloning of PCR products was performed for the H4/H3 primer design phase and for those individuals that proved to be heterozygous with respect to insertions and deletions (indels), resulting in varying spacer lengths and thus impeding direct sequencing. Nine of the 14 specimens with heterozygous sequences yielded fragments differing in spacer length. The fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN), extended by A-endings with the DyNAzyme II DNA polymerase (Finnzymes) and cloned with the TOPO TA cloning kit (Invitrogen). Two to four clones were sequenced until the two main variants were obtained. In two cases (samples WIW2_392 and MLF1_3939) more than two length variants were obtained, which is not completely unexpected in multi-copy gene families. Sequencing was also performed at LGC Genomics (Berlin) using M13 universal primers. All sequences are deposited in GenBank under the following accession numbers: KC568830–KC569204, KJ656162–KJ656172 (COI); KC569205–KC569260, KJ656173–KJ656183 (16S); KC569261–KC569327 (H4/H3).

Sequence Analysis and Phylogenetic Tree Calculation

Sequences were edited using Bioedit 7.1.3 [28]. When directly sequenced H4/H3 fragments provided ambiguous positions, the respective sites were filled with the corresponding IUPAC codes. The complete COI data set comprises 374 sequences (including a single specimen of *O. conica* as outgroup). The alignment of the 655 nucleotide sites was straightforward because there were no indels. Statistical analyses were performed using all COI sequences. Haplotype and nucleotide diversity were calculated with DnaSP

5.10 [29], and uncorrected genetic *p*-distances between the clades of *O. dolium* and *O. conica* were calculated with MEGA 5.2 [30]. For phylogenetic tree calculation, identical haplotypes from the same geographic areas (SOIUSA codes) were collapsed resulting in a total of 220 COI sequences of *O. dolium* (197 haplotypes). Prior to the phylogenetic tree inference, a search for the best fitting substitution model was performed with jModelTest 0.1 [31]. A Bayesian inference (BI) was calculated with MrBayes 3.2.2 [32,33] for 5×10^6 generations (samplefreq = 100; nruns = 2; nchains = 4), applying the parameters obtained from the model test (GTR+G+I; nst = 6, rates = invgamma). Tracer 1.5 [34] was used to assess whether the two runs had converged and when the stationary phase was reached. The first 25% of the trees were discarded as burnin and a 50% majority rule consensus tree was calculated from the remaining trees.

The 16S sequences (55 specimens including *O. conica*) were aligned with ClustalX [35] using default parameters. The original alignment contained 879 positions of which all 61 gap positions were excluded with TrimAl 1.3 [36], implemented in the Phylemon 2.0 web tools [37], using the "no gap" option. Another 84 sites were excluded by performing the "strict" option, leaving 734 positions in the final alignment for the phylogenetic tree analyses. Of those, 217 sites were variable, compared to 331 in the original and 298 sites in the "gaps excluded" alignment. The "no gap" alignment was also used for calculation of uncorrected *p*-distances. The BI was performed with two data partitions (COI and 16S "strict"), using the substitution models suggested by jModelTest 0.1 [31] (COI: HKY+I+G: nst = 2, rates = invgamma; 16S "strict": HKY+I+G: nst = 2, rates = invgamma), and allowing MrBayes to evaluate the model priors independently. A Maximum Likelihood (ML) tree was calculated with MEGA 5.2 [30], applying the sequence evolution model GTR+G (5 rate categories)+I and performing 1000 bootstrap replicates with SPR

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Table 2. Primer sets for amplification and sequencing.

Region	Primer (5′ to 3′)	Origin	Fragment size	T Phusion	T Roche
COI fwd	COIfolmer_fwd: GGTCAACAATCATAAAGATATTGG	[26]	655 bp	54°C	50°C
COI rev	H2198-Alb: TATACTTCAGGATGACCAAAAAATC	[10]			
16S fwd	16SLOrc1_fwd: TTACCTTTTGCATAATGGTTAAACTA	present study	c. 850 bp	60°C	54°C
	16SLOrc2_fwd: TTACCTTTTGCATAATGGTTAAATTA	present study			
16S rev	16SLOrc_rev: CGGTCTGAACTCAGATCATG	present study			
H4/H3 fwd	OrcH4_left1: GTGCGTCCCTGGCGCTTCA	present study	c.1100 bp	71°C	57°C
	OrcH4_left2: GGCGCTTCAGGGCGTACACT	present study			
H4/H3 rev	OrcH3_right1: TGGGCATGATGGTGACACGCT	[27]			
intern fwd	OrcH4S_left3: CGGTCTGAACTCAGATCATG	present study			
intern rev	OrcH3S_right3: CGGTCTGAACTCAGATCATG	present study			

The annealing temperatures are provided for the Finnzymes Phusion and the RocheTaq Polymerase, respectively.
doi:10.1371/journal.pone.0096012.t002

(Subtree-Pruning-Redrafting) as heuristic method for tree inference. Support values of the ML analysis are provided for the combined COI and 16S tree.

A Median-Joining network was calculated from the H4/H3 data with Network 4.6.0.0 (Fluxus Technology Ltd.) using the default settings. Two networks were calculated: one without gaps and one keeping the gap positions. The networks were then post-processed to reduce unnecessary median vectors using the MP option. As the gap positions contained valuable information, we show the network including the gap sites.

Molecular Clock Analysis and Geographic Range Reconstruction

A reconstruction of the historical biogeography of *O. dolium* was performed with Lagrange 2.0.1 [38]. Lagrange 2.0.1 uses a dispersal-extinction-cladogenesis (DEC) modeling, which allows analyzing the ML values of rate transitions as a function of time. The calculations were based on a molecular clock dated linearized BI tree calculated with BEAST 1.7.5 [39]. The fossil record of *O. dolium* comprises only material of the Holocene and the Middle and Late Pleistocene. This might be due to the fact that during these time periods climate conditions promoted the accumulation of loess sediments containing high numbers of gastropod shells. In contrast, fossil records of the Early Pleistocene and the Pliocene are very scarce and lack many land snail species endemic to Central Europe, among them *O. dolium*. Consequently, dating the stem of the tree with the species first occurrence in the fossil record was not a reasonable option. Hence, substitution rates were inferred from a comparison of two other Orculidae, *Orculella aragonica* and *O. bulgarica*. The latter is widespread in South-Eastern Europe and Western Asia, whereas its sister species, *O. aragonica*, is distributed only in the Iberian Peninsula [22]. The earliest fossil record of *O. aragonica/O. bulgarica* dates from about 1.8 mya at the Almenara-Casablanca karst complex (Castellón, Eastern Spain), which contains Miocene to Early Pleistocene sediments [40,41]. We assumed that the earliest record coincides with the time period

when the ancestral lineages of the two species split from each other. Calculation of substitution rates and molecular clock analysis were both performed with the same COI and 16S ("strict", 696 positions) alignments (including the sequences of *O. conica* and the two *Orculella* species). For each partition, TN93+G was applied both to the measurement of substitution rates and the inference of the molecular clock dated tree. Molecular clock tests were performed independently for the COI and 16S alignments with MEGA 5.2 under the TN93 model, using a discrete Gamma (G) distribution to model differences in evolutionary rates among sites. The null hypotheses of equal evolutionary rates throughout the trees were rejected at a 5% significance level. Substitution rates (COI: 0.02333, 16S: 0.02528; substitutions/ma) were assigned in the prior settings of BEAUti 1.7.5 (part of the BEAST package), and uncorrelated relaxed lognormal molecular clocks were implemented for both sequence partitions. "Speciation: Yule Process" was chosen as tree prior. The BEAST analysis was performed with four independent runs with 10^7 generations each (sample freq.: 1000). Tracer 1.5 [34] was used to assess whether the four runs had converged. The four independent runs were then combined with LogCombiner 1.7.5 (part of the BEAST package). Subsequently, 25% of the trees were discarded as burnin and a 50% majority rule consensus tree was calculated from the remaining 3×10^5 trees. Median node heights and 95% highest posterior density (95% HPD) intervals are provided for major nodes in the results section. The rate-calibrated linearized tree was then prepared for Lagrange configurator 20130526, together with a range matrix in which each lineage was assigned either to the Northern Calcareous Alps, the Southern Calcareous Alps, the Western Alps or the Western Carpathians. Migration was permitted between all regions, but lower probabilities ('0.5' instead of '1.0') were assigned in the dispersal constraints for migration between areas not being immediately adjacent (Southern Calcareous Alps and Western Carpathians; Southern Calcareous Alps and Western Alps; Western Carpathians and Western Alps). The ancestors were allowed to occupy a maximum of two geographic

Figure 2. BI analysis of COI haplotypes. The central tree provides an overview. Posterior probabilities are given at the main nodes. Black dots indicate nodes with maximum support. A specimen of *O. conica* is used as outgroup. More details are provided in the partial trees with different colors corresponding to the geographic mountain regions depicted in Fig. 1. The scale bars indicate the expected number of substitutions per site according to the model of sequence evolution applied.
doi:10.1371/journal.pone.0096012.g002

areas. Alternative analyses were run with unlimited range sizes (allowing the taxa to inhabit all four geographic areas) and assigning the same probabilities ('1.0') for migration between the four areas in the dispersal constraints.

Literature Search for Fossil Records of *O. dolium*

We screened various papers for fossil records of *O. dolium*. The dating of the Alpine sites seems to be rather tentative because all but one site were dated only using reconciliation with vertebrate fossils of the same layers. Most reliable is the stratigraphic dating of recently investigated loess profiles of the Pannonian Basin, including measurements of carbonate content variations, low-field magnetic susceptibility and radiocarbon dating using macro charcoal fragments. Most of the Western Carpathian records are based on investigations of soil profiles as well. The publications with positive records of *O. dolium* are listed according to the countries investigated: Austria [42,43], Croatia [44,45], Czech Republic [46–48], Germany [49], Hungary [19,50], Serbia [51–53] and Slovakia [47,48]. Since the evaluation of the fossil record of *Orcula dolium* was based on literature data only, no permits were required for that part of the study. We assigned each record to a time period of either cold or warm climate phases of Middle and Late Pleistocene according to the Quaternary divisions of the North European climate cycles of Zagwijn [54]: the Weichselian (115–11 ka ago), Saalian (350–130 ka ago) and Elsterian (475–370 ka ago) were considered as cold climate stages (glacials),

compared to the warm stages (interglacials), Holocene (11 ka ago - present), Weichselian-Saalian interglacial (= Eemian; 130–115 ka ago) and Saalian-Elsterian interglacial (= Holsteinian; 370–350 ka ago). However, it has to be noted that massive glaciations of the Alps and northern Europe occurred only at some times of the glacials, most recent during the LGM (30–18 kya). The maps showing the distribution of genetic clades and fossil records were prepared using ArcMap Desktop 10.0 and manually edited in Adobe Photoshop CS4 version 12.

Results

Mitochondrial Clades

Among the 373 individuals of *O. dolium* investigated, 197 COI haplotypes are observed. The phylogenetic trees calculated with different algorithms show similar topologies. Ten major clades, some of them divided into distinct sub-clades, are highly supported in all analyses. Six of the major clades occur exclusively in the Western Carpathians (2, 3, 5, 7, 9 and 10), three clades are distributed solely in the Alpine region (4, 6 and 8). The final clade, clade 1, is subdivided into four sub-clades themselves restricted to particular regions (Alps: 1A; Western Carpathians: 1B, 1C and 1D) (Figs. 2, 3 and 4). Sub-clade 1A is distributed throughout the Northern Calcareous Alps but occurs also in a distinct area of the Southern Calcareous Alps. Similarly, clade 6 is distributed in separate areas of the Northern Calcareous Alps and the Southern
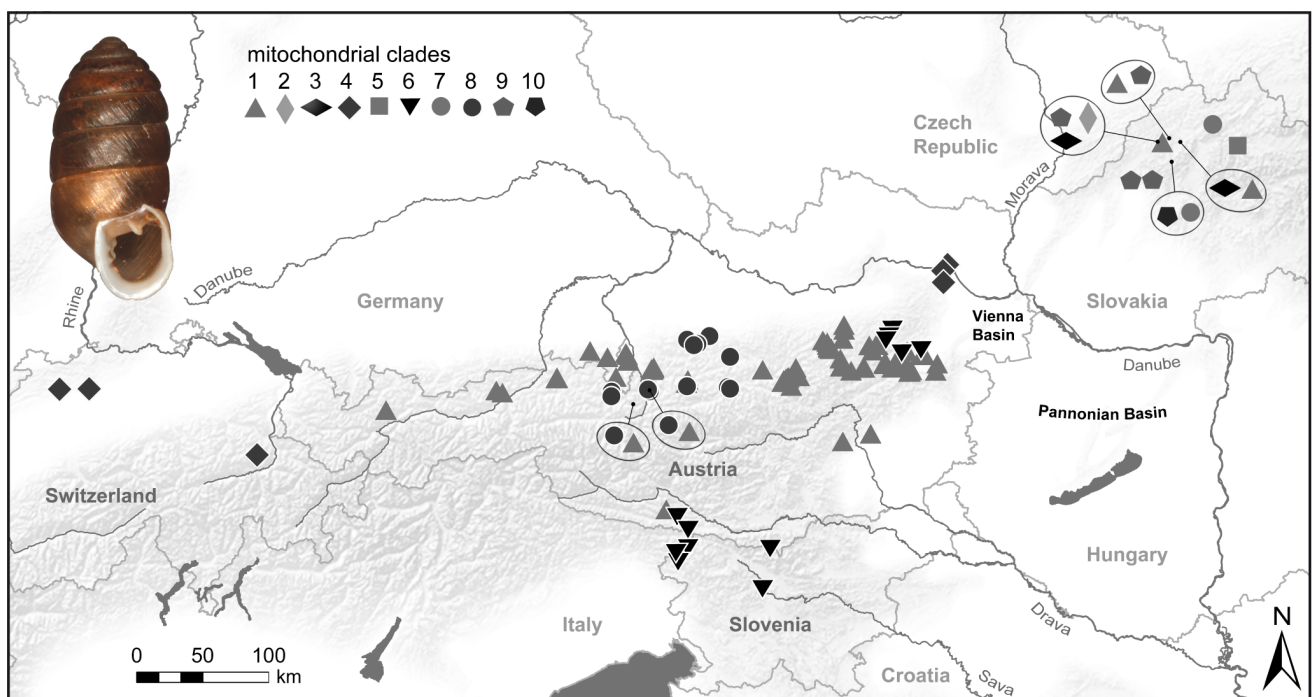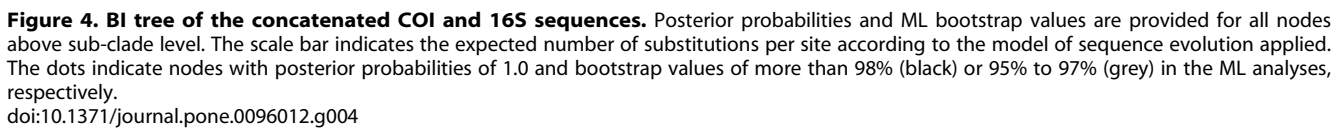

Figure 3. Distribution of localities investigated in the Alps and the Western Carpathians. The symbols indicate the presence of the respective mt clades. If specimens of different mt clades are present at the same localities, the respective clade symbols are shown encircled with a line pointing towards the locality. A picture of the shell of a type specimen of *O. dolium* (Syntype NHMW 14765/1820.26.61/2; size: 6.8 mm in height) is provided in the left upper corner.
doi:10.1371/journal.pone.0096012.g003

Figure 4. BI tree of the concatenated COI and 16S sequences. Posterior probabilities and ML bootstrap values are provided for all nodes above sub-clade level. The scale bar indicates the expected number of substitutions per site according to the model of sequence evolution applied. The dots indicate nodes with posterior probabilities of 1.0 and bootstrap values of more than 98% (black) or 95% to 97% (grey) in the ML analyses, respectively.
doi:10.1371/journal.pone.0096012.g004

Calcareous Alps. Clade 4 also has a disjunct distribution, but in an east-west orientation, occurring in the eastern-most part of the Northern Calcareous Alps (4B) and in the Western Alps (4A). In contrast, clade 8 is geographically restricted to a small area in the Northern Calcareous Alps. The BI tree based on COI and 16S reveals the same highly supported clades, sub-clades and overall topology as the COI tree, but some nodes are better supported. Nevertheless, the topology remains partly ambiguous, e.g., the relationships between clades 1, 2 and 3 as well as between 4, 5, and 6 are unresolved trichotomies.

Nuclear Data Set

The H4/H3 data is shown as a phylogenetic network (Fig. 5). It is not possible to incorporate the division of the mt clades into the

H4/H3 network because the pattern differs considerably from that obtained in the mt trees - specimens of the same mt clades often provide very different H4/H3 alleles. The general scheme of the network exhibits three frequent alleles (HT1, HT2 and HT3), each encircled by several similar haplotypes, which differ by a few substitutions or indels only. Additionally, there are several unique alleles, particularly in specimens from the Western Carpathians (e.g. in 1996_STR2, 1376_MLF5, 3952_MLF6, 3915_MLF3 and 1373_MLF7). In the Alpine populations, HT1 is the most frequent allele and, apart from a unique Western Alpine allele (differing by 2 substitutions from HT1), it is the only one found in the Western Alps and the Southern Calcareous Alps. In the Western Carpathians, HT1 is detected in a single specimen (STR1_3909) only. However, HT1 is encircled by several slightly differing

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haplotypes of both the Northern Calcareous Alps and the Western Carpathians. In contrast, HT2 and HT3 are each found exclusively in either the Northern Calcareous Alps or the Western Carpathians, respectively. Thus, all populations having HT2 additionally feature HT1 or haplotypes slightly deviating from HT1. Within the Alps, the populations of the eastern-most margin of the Northern Calcareous Alps exhibit the largest allele diversity.

Genetic Diversity

Intraspecific distances are extraordinarily high for the COI gene, with up to 18.4% mean *p*-distance between the clades (Table 3). The maximum *p*-distance within the Alpine populations is 16.9%, compared to 18.3% in the Western Carpathian ones. The mean *p*-distance between *O. dolium* and *O. conica* is only slightly higher at 18.4% (Table 3). The respective haplotype and nucleotide diversities of the COI clades are high in all populations (Table 4). Distances were also calculated for the 16S sequences (Table 5). The largest mean *p*-distance between clades of *O. dolium* is 14.4%, the distance between *O. dolium* and *O. conica* is 19.3%, indicating that the conserved parts of the 16S suffered less from saturation than COI. Regarding the nc H4/H3 sequences, the Western Carpathian populations almost consistently show larger *p*-distances (max.: H3 1.5%, H4 2.7%, Spacer 2.6%; clones excluded: H3 0.3%, H4 1.6%, Spacer 1.0%) than the Alpine ones (max.: H3 0.6%, H4 1.9%, Spacer 2.1%; clones excluded: H3 0.3%, H4 1.2%, Spacer 1.5%). The max. *p*-distances within *O. dolium* (H3 1.5%, H4 2.7%, Spacer 2.6%; clones excluded: H3 0.3%, H4 1.9%, Spacer 1.8%) are comparable to the mean distances between *O. dolium* and *O. conica* (H3 0.67%, H4 1.30%, Spacer 2.38%).

Molecular Clock Analysis and Reconstruction of the Phylogeographic Range Evolution

The linearized tree resulting from the BEAST 1.7.5 analysis was combined with a biogeographical range reconstruction using Lagrange v.20130526 (Fig. 6). For the nodes marking major splits of mt lineages, the node ages and the 95% HPD intervals (in mya) are provided. The alternative ancestral subdivision/inheritance scenarios with likelihoods of 15% or more are also indicated in the tree. As a main result, the analyses suggest that *O. dolium* originated 6.92 to 4.13 mya (95% HPD interval) around the Miocene-Pliocene boundary. However, the analyses support that the broad diversification into numerous lineages happened during the Pleistocene. According to the reconstruction of the species' geographic range history (ancestors allowed to occupy a maximum of two geographic areas/dispersal constraint for areas not immediately adjacent: '0.5'), the ancestral *O. dolium* was distributed in the Western Carpathians (Maximum Likelihood of ancestral stage at cladogenesis event: 0.83). The alternative analysis run without range restrictions (ancestors allowed to occupy all four geographic areas/dispersal constraint for areas not immediately adjacent: '0.5') predicted similar ancestral ranges but the most likely ancestral range scenarios generally obtained lower likelihood values. Moreover, alternative ancestral ranges predicted for several nodes comprised differing geographic areas (Fig. S2). Analyses run with the same range constraints as in the two previous analyses, but with differing dispersal constraint (migrations between all areas are equally likely) resulted in identical ancestral ranges for almost all nodes, only the ML values differed slightly (by a maximum of 10 percent) (data not shown).
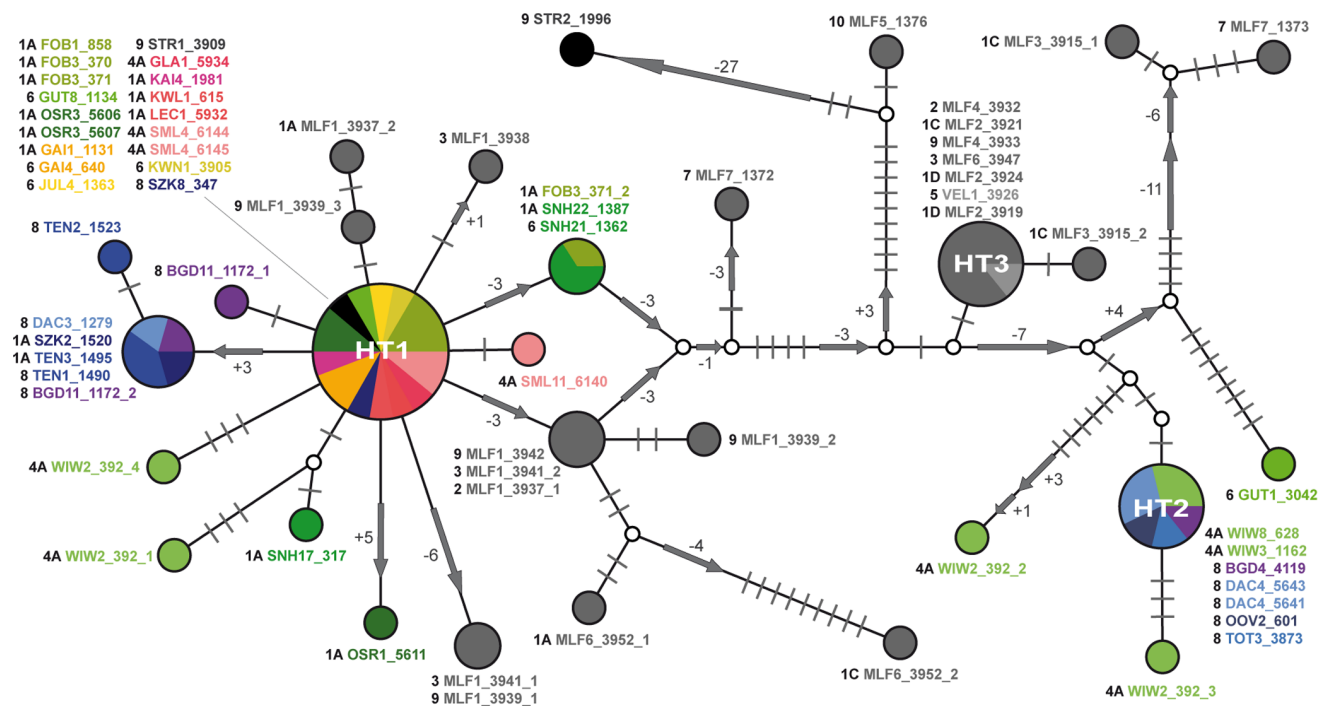

Figure 5. Median Joining network of the H4/H3 sequences. Bars indicate substitutions within the H3, H4 and the spacer region. The loss or gain of bases (indels) in the spacer region is displayed by arrows pointing in the respective direction and the numbers indicate in how many bases the haplotypes/alleles differ from each other. The three most common alleles are named as HT1, HT2 and HT3. The size of the circles corresponds to the number of sequences providing the same allele. The colors correspond to one of the mountain regions defined in Fig. 1. To facilitate the comparison of nc and mt data, the specimen labels and the clade affiliation are indicated next to the haplotype circles.
doi:10.1371/journal.pone.0096012.g005

Table 3. Mean and maximum genetic *p*-distances (in %) for the COI sequences.

mt clade	1A	1B	1C	1D	2	3	4A	4B	5	6	7	8	9	10
dist. Ø	0.9	0.1	0.9	0.5	n/c	2.2	2.6	0.2	0.4	1.2	0.8	0.7	4.1	1.3
dist. max.	2.6	0.3	1.8	0.9	n/c	4.3	3.8	0.6	0.8	2.9	1.1	1.4	6.7	1.4
1A														
1B	3.8													
1C	3.0	3.7												
1D	3.6	4.4	3.3											
2	5.1	5.6	4.8	5.8										
3	5.7	5.9	5.3	5.7	5.1									
4A	7.2	7.7	6.9	7.4	6.7	8.1								
4B	6.1	6.7	5.8	7.0	5.7	7.0	4.6							
5	7.2	7.6	6.8	7.5	5.7	7.1	6.5	5.4						
6	7.1	7.1	7.0	7.7	6.9	7.8	7.4	6.3	6.0					
7	13.5	14.3	14.2	13.6	13.6	13.4	13.6	14.5	13.4	14.8				
8	14.5	15.2	15.2	15.0	14.6	13.9	14.7	16.1	14.7	15.8	5.6			
9	15.4	15.8	15.3	15.1	16.0	15.1	15.1	15.2	15.6	15.9	16.7	16.9		
10	16.9	17.5	16.8	16.2	18.1	16.8	17.2	16.8	17.9	18.4	15.9	16.4	14.2	
O. conica	18.0	17.9	18.7	18.2	18.8	18.2	18.6	18.6	18.2	19.1	18.7	20.1	16.6	16.5

doi:10.1371/journal.pone.0096012.t003

Middle and Late Pleistocene Distribution Derived from Fossil Record

The information about the fossil distribution of *O. dolium* is displayed in Fig. 7. Separate maps are presented for cold and warm Pleistocene climate stages because several localities provide records of both glacial and interglacial periods. The literature reports *O. dolium* from about 100 fossil sites, most of which are located in the Western Carpathians, including 40 sites in Slovakia alone [47,48]. Four Czech localities provide Middle Pleistocene to Holocene fossils, among them the northern-most confirmed site, which is located about 30 km north of Prague [47]. Around 20 sites are located in the Western Carpathians of Northern Hungary, with Holocene, Weichselian, Eemian and Elsterian deposits [19,48,50]. Some of these represent the earliest fossil records of the species, particularly the sites in the Hungarian Bükk mountains and the surroundings of Budapest. Deposits from the Slovenská Skala in South-Eastern Slovakia also date back to the Elsterian and Holsteinian about 400 ka ago [48]. Fewer data are available from Austria and Germany. Most of the 18 sites of the Northern Calcareous Alps are dated to the Holocene or the Weichselian. Only a single Alpine site (Vienna, Austria) was tentatively classified to the end of the Middle Pleistocene by [42]; it is not included in the maps because an assignment to a cold or warm climate stage is not possible. The Nußloch site (Baden-Württemberg, Germany) is the earliest fossil occurrence in the foreland of the Western Alps in the Early and Middle Weichselian, with an increasing frequency in the Late Weichselian layers [49]. Numerous fossil records of *O. dolium* were reported from loess deposits of the Pannonian Basin [19,52,55], most of which are located along the Danube in southern Hungary and Northern Serbia, where the rivers Sava and Danube join. Most Pannonian sites provide Weichselian deposits, although the species was already present close to Novi Sad in Serbia at the end of the Saalian [51]. In Eastern Croatia, fossils of *O. dolium* date back from the Weichselian to the Saalian (over 200 ka ago) [44,45].

Discussion

Origin and Diversification of *O. dolium*

Zimmermann [56] hypothesized that *O. dolium* originated in the Northern Calcareous Alps like several congeneric species. Frank [42] took up the same position and stated that *O. dolium* emerged in the Northern Calcareous Alps in the Early Pleistocene. However, these assumptions regarding the species' origin are rather tentative because no Pliocene and Early Pleistocene fossils are known from Central Europe. The earliest record of *O. dolium* from South-Eastern Slovakia and North-Western Hungary dates back to the Elsterian (475–370 kya) and even earlier [19,48], whereas almost all records from Alpine sites were assigned to the Weichselian (115–11 ka ago) or the Holocene; only a single record (Austria, Vienna) was vaguely dated to the Late Middle Pleistocene [42]. In general, the gastropod record before the Middle Pleistocene is extremely scarce because sediments predominantly consist of red clay, being inappropriate for the fossilization of gastropod shells [19]. Besides, environmental dynamics in the mountainous regions, especially in the preferred limestone habitats, offer few opportunities for shell fossilization. Hence, in the present study, hypotheses regarding the origin and diversification of *O. dolium* are mainly based on molecular genetic data.

Contradicting the assumption of Zimmermann [56] and Frank [42], the variability of the mt and nc markers and the geographic distribution of haplotypes support an origin of *O. dolium* in the Western Carpathians. Seven out of ten clades occur in the Western Carpathians, including two highly diverged clades (9 and 10) which split from the basal nodes of the trees. The populations of the Alps and the Western Carpathians are not reciprocally monophyletic - lineages of both areas derive from three nodes in the mt trees each. The number of H4/H3 alleles highly diverged from the three main alleles HT1, HT2 and HT3 is larger in the Western Carpathians as well. The geographic range reconstruction supports a scenario in which the most recent common ancestor was distributed in the Western Carpathians (ML: 0.83)

Table 4. Haplotype and nucleotide diversity within clades for the COI sequences.

mt clade	1A	1B	1C	1D	2	3	4A	4B	5	6	7	8	9	10	Alp.	Carp.	total
sequence no.	218	7	9	5	1	13	5	9	4	32	3	57	7	3	321	52	373
haplotype no. (h)	111	4	7	5	1	9	5	4	4	18	3	19	4	3	157	40	197
haplotype div. (Hd)	0.983	0.714	0.917	1.000	0.000	0.910	1.000	0.583	1.000	0.938	1.000	0.882	0.810	1.000	0.987	0.985	0.990
nucleotide div. (Pi)	0.009	0.001	0.008	0.016	0.000	0.022	0.026	0.002	0.004	0.012	0.008	0.007	0.041	0.013	0.062	0.092	0.069

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around 6.26 mya (95% HPD: 5.15 to 7.42); the Northern Calcareous Alps were probably settled later (Fig. 6). A scenario in which the MRCA was distributed in both the Western Carpathians and the Northern Calcareous Alps obtained only low support (ML: 0.12). The distributional patterns of mt clades/ variants can best be explained by multiple (probably two or three) migrations between Alps and Western Carpathians with single specimens or populations carrying unique or similar mt variants, respectively. Since the Alpine clades are embedded within the diversity of the Western Carpathians, a predominant east-west migration route is most probable. Alternative to scenarios with multiple migrations, the Alpine diversity could have resulted from a single migration involving multiple individuals carrying strongly differing mt variants. However, the geographically distinct distributions of the Alpine mt clades suggest that the lineages evolved independently from each other. Moreover, the molecular clock analysis indicated that the Alpine mt clades separated from their closest related Western Carpathian lineages during different time periods. The results of the analysis suggested that the Alpine mt sub-clade 1A descended from the Western Carpathians rather recently during the Middle Pleistocene, whereas clade 8 and the cluster including clades 4 and 6 separated from their closest related Western Carpathians lineages probably during the Early Pleistocene already. The nc H4/H3 sequence patterns also support at least two independent migration events, as two highly diverged, geographically more or less separated clusters (HT1, HT2 and similar variants) were found.

Pleistocene Refuges and Postglacial Expansion Routes

One of the present study's main objectives is the detection of potential glacial refuges of O. dolium. The four major limestone areas currently inhabited by O. dolium (Western Alps, Northern Calcareous Alps, Southern Calcareous Alps and Western Carpathians) are treated separately, as is the Pannonian Basin, in which the species is apparently not found nowadays.

The extensive fossil record of the Western Carpathians, with data from both glacials and interglacials, confirms that the area was permanently settled, at least during the second half of the Middle Pleistocene and the Late Pleistocene [48]. In particular the extensive record of Weichselian (115–11 ka ago) fossils provides evidence that O. dolium was widely distributed during the last glacial (Fig. 7). Moreover, despite the comparably small sample size, the Western Carpathian populations show a large genetic diversity with complex distribution patterns. There is no clear geographic structure regarding the distribution of mt clades and nc alleles, and the data do not indicate a serious loss of genetic diversity due to genetic bottlenecks. Unlike in the Alpine region, the loss of potential habitat presumably was less significant in the Western Carpathians, which were not affected by massive glaciations during Pleistocene cold stages. The scattered distribution of limestone bedrock in the Western Carpathians is another factor, which may have triggered diversification and preservation of numerous genetic lineages.

The eastern part of the Northern Calcareous Alps potentially provided the largest Alpine refuge for calciphilous taxa because it continuously offered non-glaciated limestone areas. Patterns of endemism and comparative phylogeographic analyses in Alpine plants [8] provide additional evidence for refuges in this area. Similarly, the Northern Calcareous Alps harbor a number of endemic species such as Trochulus oreinos and Cylindrus obtusus which probably originated in that region [14]. Haplotypes of all four Alpine mt clades are found here, with populations located somewhat separated from each other, and therefore suggesting several smaller refuges. Moreover, the respective populations show

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Table 5. Mean and maximum genetic *p*-distances (in %) for the 16S sequences.

clade	1A	1B	1C	1D	2	3	4A	4B	5	6	7	8	9	10
dist. max.	1.1	–	1.6	0.1	–	3.2	3.6	0.3	–	2.4	0.8	1.1	7.0	–
1A														
1B	2.1													
1C	2.3	1.5												
1D	2.0	2.1	2.1											
2	2.9	3.0	3.1	3.2										
3	4.0	3.7	4.1	3.5	3.4									
4A	4.5	4.1	4.0	3.8	4.6	5.1								
4B	3.8	3.2	3.2	3.0	3.3	3.7	2.7							
5	4.5	4.0	4.4	3.9	3.9	4.1	5.2	4.3						
6	3.4	3.2	3.5	3.1	3.0	3.7	3.8	2.8	3.8					
7	12.2	12.0	12.5	11.9	12.7	12.8	12.7	12.4	13.2	12.7				
8	11.6	11.2	11.6	11.1	11.8	11.8	11.8	11.6	12.3	11.9	2.2			
9	13.2	12.8	13.1	12.4	13.7	13.7	13.7	13.4	13.5	13.1	13.8	13.1		
10	13.6	13.0	13.7	13.2	14.2	14.2	14.4	14.3	14.0	13.4	14.3	14.1	12.3	
O. conica	19.5	19.4	19.7	19.0	20.1	20.0	19.4	19.8	19.9	19.0	19.8	18.8	17.3	19.0

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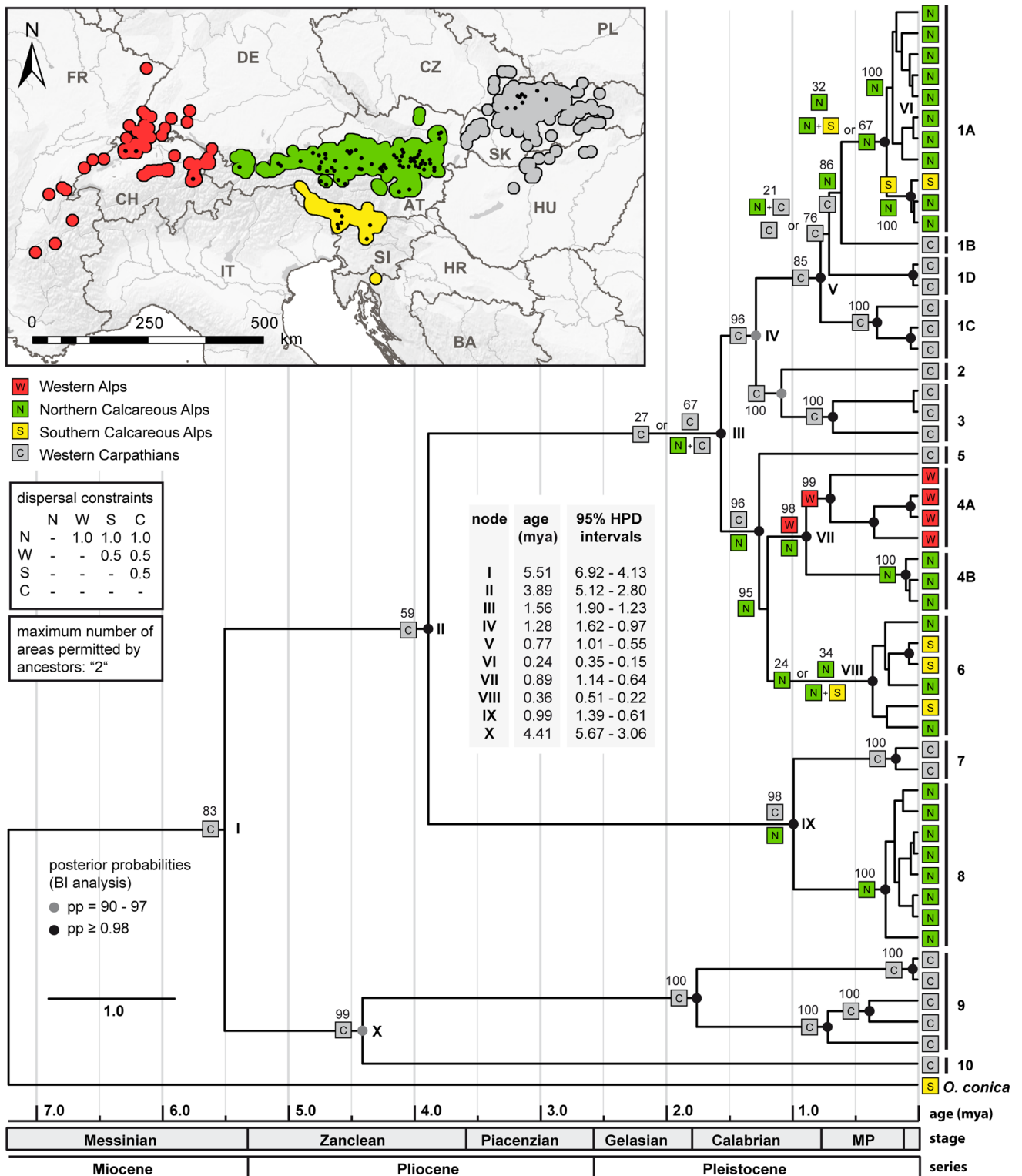
Figure 6. Reconstruction of the geographic range evolution. The map shows the distribution areas of *O. dolium* in the four Alpine and Carpathian mountain areas sampled (encoded by different colors). Small black dots represent localities sampled in the present study. The linearized molecular clock dated BI tree shows the relationships of selected mt lineages (COI/16S data) of *O. dolium*. Black and grey dots indicate nodes with high posterior probabilities (see figure for values). The colored symbols at the branch tips indicate the geographic origin of the haplotypes. The ancestors were allowed to occupy a maximum of two geographic areas. At the cladogenesis events (nodes), all alternative ancestral subdivision/ inheritance scenarios with likelihoods of 15% or more are indicated, together with the respective likelihoods, and separated by an ''or''. When scenarios for cladogenesis events involve two ancestral areas, the symbol for the likely ancestral area/−s is/are provided left to each of the two branches. For nodes representing major splits, node ages and 95% posterior HPD intervals are indicated. A time scale in mya is given below.
doi:10.1371/journal.pone.0096012.g006
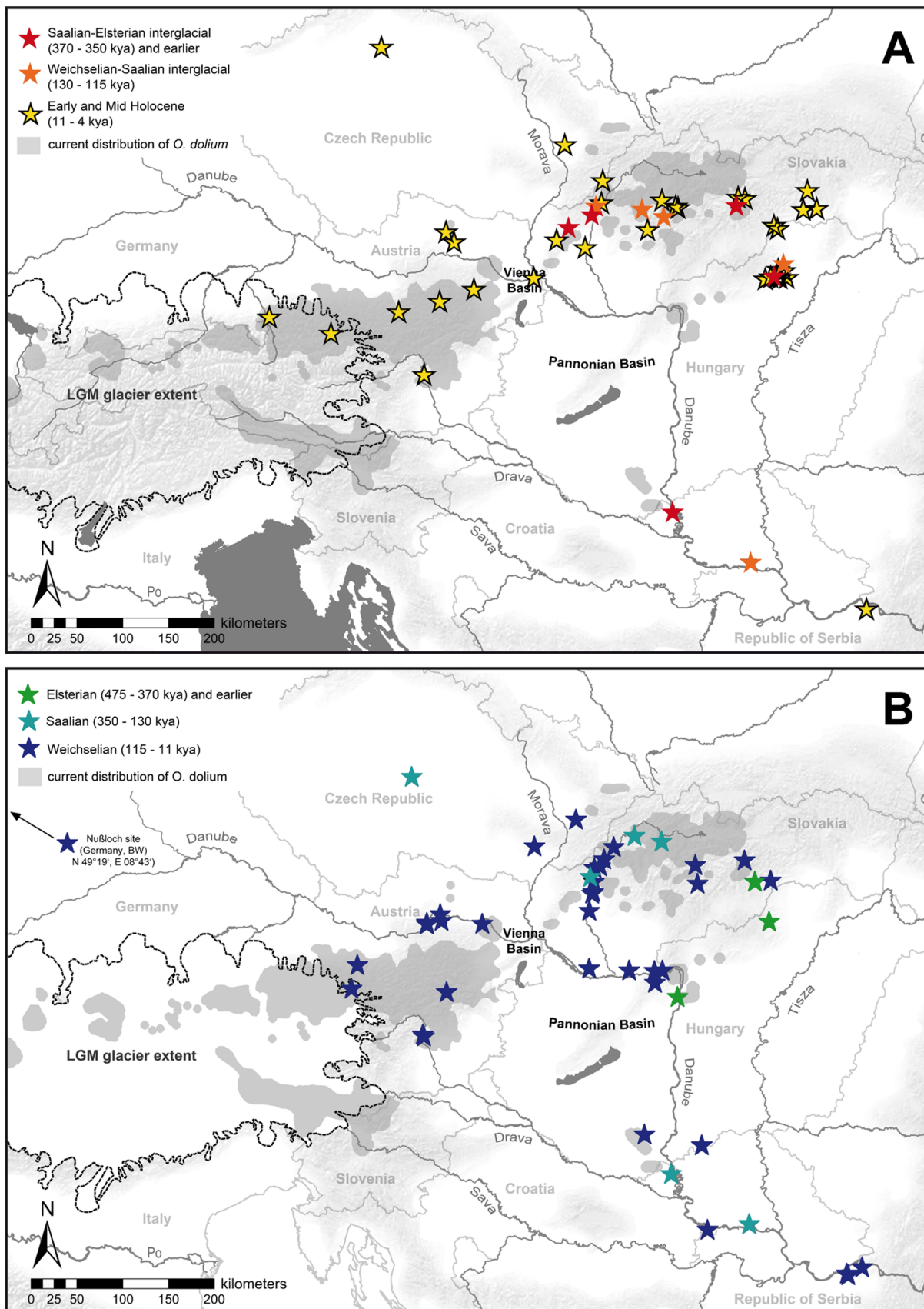
Figure 7. Distribution of fossil *O. dolium* in Central Europe during warm (A) and cold (B) Pleistocene climate stages. The LGM glacier line indicates the maximum extent of glaciers during the Weichselian (35 to 19 ka). The grey-shaded areas correspond to the current distribution of *O. dolium*.
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a high diversity in nc H4/H3 alleles. The most common mt clade (1A) is distributed from Lower Austria (Gutenstein Alps) in the east to Tirol (Lechtal Alps) in the west, spanning a distance of 400 km (Fig. 3). A distinct mt clade (4B) is present at the eastern edge of the Alps (Wienerwald), a region which is geologically somewhat isolated from the Northern Calcareous Alps due to the predominance of siliciclastics in the intermediate region. The large nc diversity with several H4/H3 alleles, each strongly diverged from the most common alleles HT1 and HT2, and the presence of Weichselian fossil deposits support the assumption that the Wienerwald served as a long-term refuge. The Gutenstein Alps at the northeastern margin of the Northern Calcareous Alps may have provided a refuge area for the population exhibiting mt clade 6. Another potential refuge was located close to the former LGM glacier line in Salzburg and Upper Austria - mt clade 8 is distributed exclusively in this region, and fossil deposits indicate the local presence of the species during the Weichselian [42]. Some of the specimens carrying haplotypes of mt clade 8 possess the nc allele HT2 or similar ones, which elsewhere occur in the Wienerwald only. This can either be explained by past gene flow between the two currently separated populations or by ancestral polymorphism, i.e., the persistence of ancestral histone variants in both areas.

The geographic range reconstruction suggests that the Western Alps were settled from the Northern Calcareous Alps during the Middle Pleistocene 1.05 to 0.66 mya (95% HPD interval). Samples from the Western Alpine sites all form a single mt sub-clade (4A) and have the nc haplotype HT1 or similar types. Although material is available from three sample sites only, the Western Alpine populations show higher distances in the mt sequences (max. uncorrected *p*-distance COI: 3.8%) than any other Alpine clade. The unique presence of the highly diverged mt clade 4A provides support for a refuge in the Western Alps. Since the Western Alps were almost completely covered by ice during several glacials, populations may have outlasted the glacial periods in several smaller refuges at the Western Alpine margins of Switzerland and France as was proposed for *Trochulus villosus* [15] or *Carychium tridentatum* [13]. Fossils in Early to Late Weichselian deposits of the Western Alpine foreland (Nußloch, Baden-Württemberg, Germany) clearly support that assumption, at least for the last glacial period [49]. We have no molecular data from the German and French areas, but a common ancestry of the western populations is supported by similar conchological traits (collection material of the Natural History Museum, Vienna and Naturmuseum Senckenberg, Frankfurt am Main; Harl et al. in prep.).

The Southern Calcareous Alps were almost completely covered by glaciers during the LGM and hence provided only a small potential refuge for calciphilous taxa in the eastern-most part: a small non-glaciated area in the Karawanks [57]. The phylogenetic data argue against a permanent refuge of *O. dolium* in the Southern Calcareous Alps and suggest that clades 1A and 6 probably reached this region rather recently. In the Southern Calcareous Alps, sub-clade 1A is found at a single site (GAI1) only, with haplotypes similar to those in populations of the Tirolian and Bavarian Northern Calcareous Alps. Since both areas were fully glaciated, clade 1A might have crossed the Central Alps in this area after the LGM. Apart from this single locality, all other sites of the Southern Calcareous Alps possessed haplotypes of clade 6, which is distributed in the Northern Calcareous Alps as well. The similarity of clade 6 haplotypes rather indicates a very recent expansion during the Late Pleistocene or Holocene. However, the presence of a distinct variant of clade 6 at a single site in the

Karawanks (KWN1) could be an indication for a Southern Calcareous Alpine refuge.

The fossil record indicates a more or less continuous presence of *O. dolium* in the Pannonian Basin at least from the Saalian onwards (over 200 ka ago). The habitats of the Pannonian populations were probably patchily distributed forests near rivers, which were present in the area even during the LGM [7]. The occurrence of trees at the respective sites is additionally supported by the co-occurrence of other woodland species in the same loess strata, such as *Semilimax semilimax*, *Ena montana* or *Aegopinella ressmanni* [45,58]. One might ask whether riparian drift from Alpine or Western Carpathian regions could account for the presence of *O. dolium* in the fossil record of the southern part of the Pannonian Basin. However, the high abundance of fossil *O. dolium* in the Pannonian Basin indicates a local source. The contemporary absence of the species is probably the result of anthropogenically induced loss of suitable habitat. Ložek [48] stated that deforestation and dehydration are probably the reasons why these areas lack several gastropod species which were still widely distributed during the Eemian. Thus, the expansion of agricultural areas is a reasonable explanation for the decline of *O. dolium* populations in the Pannonian Basin during the Holocene.

Genetic Differentiation and Taxonomic Considerations

The intraspecific distances measured for the mt genes are among the highest found in pulmonate species (uncorrected *p*-distances: COI, 18.4%; 16S, 14.4%). By comparison, the genetically diverse helicid taxa *Theba pisana* and *Arianta arbustorum* show COI distances of 13.6% [59] and 15% [10], respectively. Regarding the non-protein coding 16S, higher intraspecific distances were found in the clausiliid species *Charpentieria itala* with 24.2% [60]. 16S divergence is also high in the bradybaenid *Euhadra quaesita* with 14.1% [61]. Regarding the nc sequences analyzed, the largest *p*-distance measured within the protein coding H4 and H3 sequences is 0.8%. The highest distance observed in the non-protein coding spacer region is 1.8%. [59] reported *p*-distances of 0.5% in the non-coding ITS1 sequences of *Theba pisana*, whereas the intraspecific sequence divergence within *Arion subfuscus*, a species extremely variable in its mtDNA, is only 0.3% for the ITS1 sequence [62].

Considering the large genetic variability found within the populations of *O. dolium*, the question arises whether some of the lineages might even represent distinct species. However, there are no indications of hybridization barriers that would suggest splitting the groups into different species. The data indicate gene flow between clades, as suggested by the fact that the specimens displaying the main histone gene variants each feature haplotypes of very distant mt clades (see Fig. 5). However, whether genetic groups correspond to currently accepted subspecies remains a problematic issue. More than 20 subspecies have been described for *O. dolium*, equally divided between the Alps and the Western Carpathians [20]. Most were characterized by minor differences in shell shape and the formation of the aperture folds. Our study includes specimens from several type localities of Slovakian subspecies, namely of *O. d. titan* Brancsik, 1887, *O. d. brancsikii* Clessin, 1887, *O. d. minima* Brancsik, 1887, and *O. d. cebratica* Westerlund, 1887 (Table 1 and Fig. S1). However, none of the clades can be definitively attributed to one of these subspecies. For instance, populations of the very slender, large-shelled *O. d. brancsikii* share the same mt haplotypes (clade 1C), and the nc haplotype HT3, with compact, small *O. d. minima* morphs. The very large-shelled *O. d. titan* possesses a diverged mt haplotype within clade 9 but shows the most common nc haplotype HT1, which otherwise is found in the Alps only. The Slovakian *O. d.*

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cebratica (clade 5) clusters with the Alpine mt clades 4 and 6 but displays a distinct nc haplotype (HT3). Despite the extremely high conchological and genetic variability in the Western Carpathians, we could not detect a clear correspondence between genetic haplotype groups and subspecies ranges defined by [17]. The geographically isolated and conchologically aberrant endemic of the Wienerwald, *O. dolium infima*, is genetically differentiated from other populations in the mt trees (sub-clade 4B). In contrast, individuals from the type localities of *O. dolium gracilior* Zimmermann, 1932, *O. dolium pseudogularis* Wagner, 1912, *O. dolium edita* Ehrmann, 1933, and *O. dolium raxae* Gittenberger, 1978, all possess haplotypes of the homogeneous mt clade 1 and exhibit the nc haplotypes HT1 or its derivatives, none of them forming a distinct sub-clade. The latter two taxa, *O. d. edita* and *O. d. raxae*, initially were of special interest for our study because they were reported to occur only at high altitudes [17,56]. Nonetheless, specimens from the corresponding localities do not differ from the common types of the surrounding lowlands in the markers analyzed. However, for final taxonomic decisions, the most important question is whether the presumed morphological distinctness of the various subspecies can be confirmed by morphometric investigations. Such analyses are under way to quantify morphological differences and to focus on the effect of altitude on shape formation and shell size (Harl et al., in prep.).

Supporting Information

Figure S1 Pictures of selected types of *O. dolium* subspecies. Specimens collected at the type localities of the respective subspecies were investigated in the present study. The types shown shall rather be considered as examples for the species' variability than defined discrete entities of morphologically separated populations. Some of the types indeed represent extreme morphs but transitional forms are found in most populations. The pictures were already published by Harl et al. (2011) together with data on all other currently known subspecies. In the following we provide the collection data of the specimens shown: A: *O. d. dolium* (syntype NHMW 14765/1820.26.61/2), B: *O. d. edita* (syntype LML ALT/5319/1), C: *O. d. raxae* (syntype LML ALT/5354/1), D: *O. d. pseudogularis* (syntype NHMW 56158), E: *O. d. gracilior* (syntype LML ALT/5343), F: *O. d. infima* (syntype LML/ ALT5353/1), G: *O. d. brancsikii* (syntype ? NHMW J. N. 22075), H: *O. d. titan* (syntype NHMW 68377 (5448)/3), I: *O. d. cebratica* (syntype MNHG Wstld2090), J: *O. d. minima* (syntype 27044/2). Abbreviations for Museums: NHMW (Naturhistorisches Museum,

Wien), LML (Oberösterreichisches Landesmuseum, Linz) and MNHG (Naturhistoriska Museum, Göteborg). The scale bar indicates 5 mm.
(TIF)

Figure S2 Reconstruction of the geographic range evolution. The map shows the distribution areas of *O. dolium* in the four Alpine and Carpathian mountain areas sampled (encoded by different colors). Small black dots represent localities sampled in the present study. The linearized molecular clock dated BI tree shows the relationships of selected mt lineages (COI/16S data) of *O. dolium*. Black and grey dots indicate nodes with high posterior probabilities (see figure for values). The colored symbols at the branch tips indicate the geographic origin of the haplotypes. The ancestors were allowed to occupy all four geographic areas. At the cladogenesis events (nodes), all alternative ancestral subdivision/ inheritance scenarios with likelihoods of 15% or more are indicated, together with the respective likelihoods, and separated by an "or". When scenarios for cladogenesis events involve two or more ancestral areas, the symbol for the likely ancestral area/−s is/are provided left to each of the two branches. For nodes representing major splits, node ages and 95% posterior HPD intervals are indicated. A time scale in mya is given below.
(TIF)

Author Contributions

Conceived and designed the experiments: JH MD LK HS EH. Performed the experiments: JH LK. Analyzed the data: JH LK EH. Contributed reagents/materials/analysis tools: JH MD LK HS EH. Wrote the paper: JH EH. Participated in the design and coordination of the study: MD LK HS EH. Carried out the molecular genetic studies, sequence analyses, phylogenetic analysis: JH. Created the graphics: JH. Participated in the sequence analyses: LK. Contributed in drafting the manuscript: EH. Drafted the manuscript: JH. Participated in the field work: JH MD LK HS EH. Approved the final manuscript: JH MD LK HS EH.

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Concluding Discussion

The three original papers constituting my doctoral thesis deal with various aspects of the taxonomy and evolutionary history of the land snail genus *Orcula*. The evaluation of the current taxonomic situation of the whole genus was the basis for the molecular genetic and morphological studies. The investigations on the phylogeography of the genus *Orcula* and of *O. dolium* in particular, were in the focus of my doctoral thesis. We were interested in the phylogenetic relationships of species within the genus and in patterns of temporal and geographic evolution. Matters of high interest were in particular the identification of Pleistocene glacial refuges of the Alpine species and the detection of potential hybridization events.

Glacial refuges of the Alpine *Orcula* species

The phylogeographic and phylogenetic studies on species of the genus *Orcula* constitute the main part of my doctoral thesis. A central theme in both studies is the search for Pleistocene glacial refuges of the Alpine *Orcula* species. *O. dolium*, the genus' most widespread species, inhabits the Alps and the Western Carpathians as well as surrounding lowland areas. It usually lives on limestone rock with patches of vegetation or in boulder fields in mountains up to 2200 meters above sea level. However, in lowlands its habitat also includes woodland areas on bedrock containing low amounts of limestone. The Late Pleistocene fossil record evidences the species' presence in the Pannonian Basin (Hungary, northern Serbia) along the rivers Danube and Save during several warm and cold glacial periods (e.g., Fűköh et al., 1995; Marković et al., 2008). The data do not indicate that the distribution of *O. dolium* in the latter area was smaller during glacials (summarized in **Harl et al., 2014a**). In Germany, it was present in the north of Baden-Württemberg at least during the last glacial (Moine et al., 2005). We found that the Eastern Alpine and Western Carpathian populations are genetically extremely diverse, not indicating a severe loss of genetic variability during the Last Glacial Maximum and earlier glacial maxima (**Harl et al., 2014a**). However, the distribution of Alpine mitochondrial clades indicates that populations were restricted to several smaller glacial refuges at the periphery of the Eastern and Western Alps. In the Western Carpathians we did not find such a pattern, haplotypes of different clades were present at several sites. Hence, we assume that *O. dolium* survived the Pleistocene cold stages in the Western Carpathians and in several smaller refuges at the periphery of the Alps as well as in surrounding lowlands (**Harl et al., 2014a**). In contrast, most other *Orcula* species are restricted to small mountain areas at the periphery of the Eastern Alps. Of these, *O. gularis* and *Orcula austriaca* Zimmermann, 1932 are the only two species with populations in both the Northern and the Southern Calcareous Alps, the other species each inhabit either one of the latter two regions and are usually restricted to a few mountains only. The Alpine endemics are all strictly calciphilous and require rock surfaces or boulder fields as habitats but, unlike *O. dolium*, they do not inhabit

lowland areas. Consequently, for the *Orcula* species endemic to the Alps, survival outside mountain regions was not an option during Pleistocene cold stages. Major parts of their current distribution areas are located at the periphery of the Alps, outside the area covered by glaciers during the Last Glacial Maximum, and we assume that they largely match with their glacial refuges. Intraspecific distances in the mitochondrial DNA and number of unique haplotypes are high within most of these species, and the molecular clock analyses indicate that they might have emerged already before the Pleistocene (**Harl et al., 2014b**). Unfortunately, for most species restricted to Alpine mountain regions, glacial refugia can be identified only indirectly by DNA sequence data - fossils of gastropods from the Alps are extremely rare and mostly of Holocene origin (Frank, 2006). Since Alpine mountain regions are exposed to harsh environmental influences and erosion, they do not provide favorable conditions for fossilization. In contrast, neighboring lowland areas such as the Pannonian Basin (Hungary, Serbia) and the Rhine-Valley (Germany) are covered by huge loess layers, which are rich in Late Pleistocene land snail fossils. These loess sediments predominantly contain lowland species associated with wetlands, but among the most frequent species are a few generalists such as *Arianta arbustorum* (Linnaeus, 1758), *Clausilia dubia* Draparnaud, 1805 and *O. dolium*, which are common also in various mountain habitats of the Alps and the Western Carpathians. The high genetic diversity found within Alpine populations of the latter species strongly suggests survival in Alpine glacial refuges (Haase et al., 2013; **Harl et al., 2014a**; Jaksch et al. in prep), but the fossil record evidences that additional refuges were located in the surrounding lowlands (**Harl et al., 2014a**).

Systematics of the genus *Orcula*

In the study on the genus *Orcula*, we analyzed material of all 13 extant *Orcula* species and set up phylogenies, which reliably depict the relationships between most of the species. So far, in particular Gittenberger (1978) made considerations about the relationships of *Orcula* species. He assumed a close relation between *O. gularis*, *Orcula tolminensis* Wagner, 1912, *O. pseudodolium* and *O. austriaca* and demarcated the latter group from *Orcula spoliata* (Rossmässler 1837), *Orcula restituta* (Westerlund, 1887), *Orcula conica* (Rossmässler, 1837) and *Orcula fuchsi* Klemm, 1967, which he considered as rather independent lineages. Generally, Gittenberger's assumptions were confirmed in the molecular genetic analyses performed in one of the present studies (**Harl et al., 2014b**). A different phylogenetic scheme was proposed recently by Schileyko (2012) who, based on the formation of the reproductive system, divided the species investigated into two separate species complexes, the first containing *O. conica*, *O. fuchsi*, *O. restituta*, *O. spoliata* and *O. dolium* (clear boundary between vas deferens and epiphallus), the second containing *O. gularis*, *O. austriaca*, *O. tolminensis*, *O. wagneri* and *O. schmidtii* (no visible boundary between vas deferens and epiphallus). However, a clear distinction of these groupings was not possible with the characters used by Schileyko (2012) because specimens of *Orcula dolium* were classified into both groups. Independently of these two groups, Schileyko (2012) mentioned five species groups differing in their genital anatomy and shell characteristics. In his

summarizing phylogenetic scheme the most basal species is *O. conica* and the second is *O. fuchsi*. Altogether, the results of my doctoral thesis are not in accordance with Schileyko's division, and it seems that the anatomical traits used are not suitable to deduce the phylogenetic relationships within the genus *Orcula*. The phylogenetic trees calculated with both the mitochondrial and nuclear data sets clearly support a division into three reciprocally monophyletic groups (**Harl et al., 2014b**) corresponding to the three subgenera described by Páll-Gergely et al. (2013). The subgenus *Orcula* contains all nine species showing an Alpine distribution, including the Alpine-Western Carpathian *O. dolium* and the Alpine-Dinarid *O. conica*. The subgenus *Illyriobanatica* includes the two Dinarid species, *O. wagneri* Sturany, 1914 and *O. schmidtii* (Küster, 1843), and the Southern Carpathian *O. jetschini* (Kimakowicz, 1883). The third subgenus, *Hausdorfia*, is monotypic with *O. zilchi* Urbański, 1960 from the Western Black Sea region only. In the phylogenetic trees, *O. zilchi* branches of from the basal node of the trees. The subgenera *Orcula* and *Illyriobanatica* are closest relatives in both the mitochondrial and the nuclear trees, however, the nodes obtained only low support (**Harl et al., 2014b**).

Hybridization within the subgenus *Orcula*

The mitochondrial sequences allowed distinguishing all of the *Orcula* species endemic to the Alps except for *O. gularis* and *O. pseudodolium*. Although the latter two species formed separated clusters in the morphometric plots and provided distinct nuclear histone alleles, a differentiation from each other was not possible by their mitochondrial DNA (**Harl et al., 2014b**). Our findings provide new support for speculations that *O. pseudodolium* and *O. gularis* might hybridize with each other. However, contradicting the assumptions of Wagner (1912) and other authors (Zimmermann, 1932; Klemm, 1974), who hypothesized that *O. pseudodolium* is a hybrid between *O. dolium* and *O. gularis*, we found no evidence that *O. dolium* was involved in hybridizations with any other *Orcula* species. The molecular genetic patterns only indicate that *O. pseudodolium* and *O. gularis* hybridized with each other (**Harl et al., 2014b**). Gittenberger (1978) already noted that *O. pseudodolium* cannot simply be regarded as a link between *O. dolium* and *O. gularis,* but features genital anatomical traits differing from the other two species. Surprisingly, in three *O. gularis* specimens from three separate sites of the Ennstal Alps (Styria, Austria) we found different mitochondrial haplotypes, which clustered with another species, *O. tolminensis*. As the latter species is morphologically most similar to *O. gularis*, these distinct haplotypes might represent original mitochondrial variants of *O. gularis*, which were displaced by those of *O. pseudodolium* after hybridization. Although there is good evidence that *O. pseudodolium* and *O. gularis* hybridized, we think that the problem is still far from being resolved. Shedding more light on the topic would require much denser sampling in the potential hybridization zones and analyzing more nuclear sequences.

Methodological Considerations

Molecular genetic techniques were essential for resolving the questions addressed in two of my publications. The mitochondrial cytochrome oxidase subunit I gene (*COI*) was used to infer patterns of genetic diversity in populations of *O. dolium* and the *Orcula* species endemic to the Eastern Alps. We sequenced a section of the *COI* in 624 *Orcula* specimens from 224 localities and eleven specimens of outgroup species from eight localities. The results revealed complex phylogeographic patterns in the genus *Orcula*, which allowed drawing conclusions about potential refuge areas and provided evidence for hybridization (**Harl et al., 2014a**). However, the limitations of the *COI* as a marker sequence became apparent when used for phylogenetic purposes - the sequence saturation in the *COI* sequences did not allow resolving the relationships between species. Hence, we additionally analyzed sections of two other mitochondrial markers, the 12S ribosomal RNA (*12S*) and the 16S ribosomal RNA (*16S*) genes, in 86 *Orcula* specimens and all eleven outgroup specimens. For both markers I designed primers allowing the amplification of fragments of up to 725 bp and 890 bp for *12S* and *16S*, respectively. The *12S* primers target gastropods in general and were first published in our study on Ariantinae (Pulmonata, Helicidae) (Cadahía et al., 2014) and they were successfully used also in studies of Hygromiidae (Kruckenhauser et al., 2014), Planorbiidae (Harl et al., in prep) and Clausiliidae (Fehér et al., in prep). The *16S* primers are more specific and allow amplifying a section of up to ~ 890 bp in Orculidae - they were first published in the paper on *O. dolium* (**Harl et al., 2014a**). The sequence section proved to be highly informative for resolving intrageneric relationships within *Orcula*. Usually, a section of only 450 bp was amplified in gastropods, which corresponds to the second half (3'-part) of the *16S* amplicon used in my doctoral thesis. The main reason complicating the design of universal primers for larger fragments is the high sequence variability in the 5'-section of the *16S*. Hence, primers have to be customized individually for different systematic groups. *16S* sections of similar lengths as used for my doctoral thesis were yet analyzed in studies of the Eastern Asian genus *Mandarina* Pilsbry, 1894 (Stylommatophora, Bradybaenidae) (Chiba, 1999; Davison & Chiba, 2006) and Australian Helicarionidae, Euconulidae and related groups (Hyman et al., 2007).

An important question was whether patterns in the mitochondrial and nuclear trees correspond to each other. Nuclear marker sequences previously used for molecular genetic analyses of land snails are in particular the ribosomal RNA genes *18S* and *28S* (e.g., Colgan et al., 2007; Klussmann-Kolb et al., 2008). The *18S* and *28S* sequences, however, are highly conserved and therefore less suitable for population genetics, and phylogeographic and phylogenetic studies at genus level. Less conserved nuclear markers used are the internal transcribed spacer 1 (*ITS1*) (e.g., Uit De Weerd & Gittenberger, 2004; Greve et al., 2010; Pfenninger et al., 2010), separating the *18S* and *5.8S* rRNA genes, and the histone *H3* gene (de Weerd & Gittenberger, 2013; Weigand et al., 2013). *ITS1* and *H3* proved to be more variable, but the information content is low nonetheless, because both markers are short in length. For the two phylogenetic papers of my doctoral thesis, I designed primers allowing the amplification of almost

the entire histone *H4*/*H3* sequence cluster. The histone genes are arranged in repeated clusters on the genome of eukaryotes. However, gene order, composition and orientation vary between different organisms, e.g., in mussel species of the genus *Mytilus* Linnaeus, 1758 the histone cluster includes genes in the following arrangement: *H4*, *H2B*, *H2A*, *H3* and *H1* (Eirín-López et al., 2004). Armbruster et al. (2005) first aimed at sequencing the histone cluster in land snails and found that *H4* and *H3* genes were separated by a non-transcribed spacer region in taxa of the informal group of Orthurethra sensu Bouchet et al. (2005). Their attempts to amplify the histone cluster in non-Orthurethra taxa were successful in *Trochulus villosus* (Draparnaud, 1805) and in *Succinea putris* Linnaeus, 1758 only. Armbruster et al. (2005) found that in Orthurethra taxa, the *H4* and *H3* genes are oriented in the opposite direction, a pattern verified so far in histone gene clusters of corals (Miller et al., 1993), ciliates (Bernhard & Schlegel, 1998) and *Drosophila* flies (Baldo et al., 1999). In the study of Armbruster et al. (2005), primers were set close to the intermediate spacer region wherefore the resulting marker sequence included only small parts of *H4* and *H3*. We placed the new primers close to the 3'-ends of the two coding regions (outer parts in the histone complex). The primers allow the amplification of 271 bp (of a total of 309 bp) and 347 bp (of a total of 408 bp) of *H4* and *H3*, respectively as well as the complete intermediate spacer region (243 bp to 490 bp in *Orcula*). The nuclear sequences were successfully sequenced in the same specimens analyzed for all three mt markers (*12S*, *16S*, *COI*) and showed to be highly informative. The publication on *O. dolium* was the first population genetic study for which sequences of the *H4*/*H3* histone cluster (including the spacer region) were used.

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JH conceived the study, created the graphics and drafted the manuscript. HS participated in drafting the manuscript and assisted in collecting literature data. AS conceived the study and drafted an early version of the manuscript. All authors read and approved the final manuscript.

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JH conceived the study, carried out the molecular genetic studies and sequence analyses, collected the data for the morphometric analyses, created the graphics and drafted the manuscript. BP provided essential samples and participated in drafting the manuscript. SK performed the statistic part of the morphological analyses. MD participated in the design and coordination of the study. LK participated in the design and coordination of the study. HS conceived the study and participated in its design and coordination. EH conceived the study, participated in its design and coordination and essentially helped to draft the manuscript. All authors participated in the fieldwork, read and approved the final manuscript.

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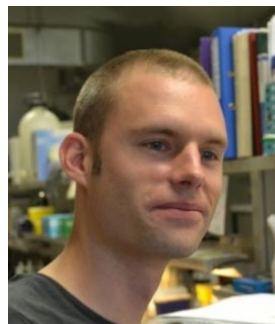
Education

07/2014 - 03/2015	Project staff at the Institute of Parasitology (University of Veterinary Medicine, Vienna) within the Austrian Barcode of Life (ABOL) project (parasitic helminths)
04/2014 - 06/2014	Work contract (database management) at the Geological-Paleontological Department (Natural History Museum Vienna)
01/2014 - 03/2014	Work contract at the University of Vienna in the course of the PhD Completion Grant 2013
01/2012 - 09/2013	Laboratory technician at the Central Research Laboratories (NHMW)
since 03/2009	Ph.D. thesis at the Central Research Laboratories (NHMW) and the Department of Integrative Zoology (University of Vienna)
09/2008 – 02/2009	Project staff at the Central Research Laboratories (NHMW)
2007 – 05/2008	Diploma thesis at the Department of Terrestrial Biology (University of Vienna) Title: 'Untersuchungen zur Ameisenfauna (Hymenoptera: Formicidae) des Nationalparks Thayatal'
2001 – 2008	Studies of Ecology and Zoology at the University of Vienna
2000 – 2001	Civil service 'Sonderkrankenanstalt Rehabilitationszentrum Saalfelden'
1991 – 1999	AHS Saalfelden (Salzburg, Austria)

Academic Teaching

2010	Co-lecturer of the practical course: 'Molecular phylogeny based on museum specimens' (University of Vienna)
2009	Co-lecturer of the practical course: 'Research training in evolutionary biology' (University of Vienna)

Awards

2013	PhD Completion Grant 2013 (University of Vienna)
2012	Theodor-Körner-Preis zur Förderung von Wissenschaft und Kunst. Title: 'Auswirkungen der Eiszeiten auf Verbreitung und Artbildung in der Landschneckengattung *Orcula* Held 1837'
2009	3. Österreichischer Nationalparkforschungspreis. Title: 'Untersuchungen zur Ameisenfauna des Nationalpark Thayatals'

Attendance of scientific meetings

2014	7th Annual Meeting of NOBIS Austria, Munich (Germany)
2014	48th Annual Meeting of the Austrian Society of Tropical Medicine, Parasitology and Migration Medicine, Graz (Austria)
2014	Workshop Alpine Landsnails, Johnsbach (Austria)
2013	World Congress of Malacology 2013, Ponta Delgada (Azores)
2013	2nd European BioSyst Meeting, Vienna (Austria)
2012	6th Annual Meeting of NOBIS Austria, Klagenfurt (Austria)
2011	5th Annual Meeting of NOBIS Austria, Salzburg (Austria)
2010	4th Annual Meeting of NOBIS Austria, Graz (Austria)
2010	World Congress of Malacology 2010, Phuket (Thailand)
2010	Workshop Alpine Landsnails, Johnsbach (Austria)
2009	3rd Annual meeting of NOBIS Austria, Vienna (Austria)
2009	102nd Annual meeting of the "Deutsche Zoologische Gesellschaft", Regensburg (Germany)
2009	1st European BioSyst Meeting, Leiden (The Netherlands).
2008	2nd Annual meeting of NOBIS Austria, Innsbruck (Austria).

Publication list

Hinney B, Ederer C, Stengl C, Wilding K, Štrkolcová G, **Harl J**, Flechl E, Fuehrer H-P, Joachim A (2015) Enteric protozoa of cats and their zoonotic potential - a field study from Austria. *Parasitology Research*, **114**, 2003-2006. **IF: 2.24**

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Zittra C, Kocziha Z, Pinnyei S, **Harl J**, Kieser K, Laciny A, Eigner B, Silbermayr K, Duscher G G, Fok É, Fuehrer H-P (2014) Screening blood-fed mosquitoes for the diagnosis of filarioid helminths and avian malaria. *Parasites & Vectors*, **8**, 16. **IF: 3.25, IF (5 year): 3.07**

Harzhauser M, Neubauer T A, Georgopoulou E, **Harl J** (2014) The Early Miocene (Burdigalian) mollusc fauna of the North Bohemian Lake (Most Basin). Bulletin of Geosciences, **89**, 819-908. **IF: 1.50, IF (5 year): 1.14**

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Duda M, Kruckenhauser L, Sattmann H, **Harl J**, Jaksch K, Haring E (2014) Differentiation in the *Trochulus hispidus* complex and related taxa (Pulmonata: Hygromiidae), Morphology, ecology and their relation to phylogeography. *Journal of Molluscan Studies*, **77**, 30-40. **IF: 1.50, IF (5 year): 1.34**

Fuehrer H-P, Swoboda P, **Harl J**, Starzengruber P, Habler V E, Bloeschl I, Haque R, Matt J, Khan W A, Noedl, H (2014) High prevalence and genetic diversity of *Plasmodium malariae* and no evidence of *Plasmodium knowlesi* in Bangladesh. *Parasitology Research*, **113**, 1537-1543. **IF: 2.85, IF (5 year): 2.51**

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Kruckenhauser L, Duda M, Bartel D, Sattmann H, **Harl J**, Kirchner S, Haring E (2014) Paraphyly and budding speciation in the hairy snail (Gastropoda, Pulmonata, Hygromiidae). *Zoologica Scripta*, **43**, 273–288. **IF: 2.92, IF (5 year): 3.01**

Cadahía L, **Harl J**, Duda M, Sattmann H, Kruckenhauser L, Fehér Z, Zopp L, Haring E (2014) 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*, **52**, 163–169**. IF: 1.91, IF (5 year): 1.77**

Fuehrer H-P, Biro N, **Harl J**, Worliczek H L, Beiglböck C, Farkas R, Joachim A, Duscher G G (2013) Molecular detection of Theileria sp ZS TO4 in red deer (*Cervus elaphus*) and questing *Haemaphysalis concinna* ticks in Eastern Austria. *Journal of Veterinary Parasitology*, **197**, 653-657. **IF: 2.381, IF (5 year): 2.611**

Páll-Gergely B, Deli D, Irikov I, **Harl J** (2013) Subgeneric division of the genus *Orcula* Held 1837 with remarks on Romanian orculid data (Gastropoda, Pulmonata, Orculidae). *ZooKeys*, **301**, 25–49**. IF: 0.864, IF (5 year): 0.902**

Fuehrer H-P, Habler V E, Fally M A, **Harl J**, Starzengruber P, Swoboda P, Bloeschl I, Wasif A K, Noedl H (2012) *Plasmodium ovale* in Bangladesh: Genetic diversity and the first known evidence of the sympatric distribution of *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* in southern Asia - International Journal for Parasitology, **42**, 693-699. **IF: 3.64, IF (5 year): 3.72**

Harl J, Sattmann H, Schileyko A (2011) Types of the extant taxa of the landsnail genus *Orcula* Held 1837 (Gastropoda: Stylommatophora: Orculidae). *Archiv für Molluskenkunde - International Journal of Malacology*, **140**, 175-199. **IF: 0.450**

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. **IF: 1.358, IF (5 year): 1.430**

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

Harl J (2010) Untersuchungen zur Ameisenfauna (Hymenoptera, Formicidae) des Nationalparks Thayatal. *Wissenschaftlichen Mitteilungen aus dem Niederösterreichischen Landesmuseum*, Band **21** St Pölten.