First record of *Trochulus clandestinus* (Hartmann, 1821) in Austria (Gastropoda: Eupulmonata: Hygromiidae)

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The north-west alpine distributed hairy snail *Trochulus clandestinus* (Hartmann, 1821) was recorded for the first time from Austria. Two living specimens were found in Vorarlberg 11 July 2016. The animals were subjected to genetic barcoding and their genital organs were dissected. The taxonomic situation within north-west alpine species of the genus *Trochulus* is not unambiguously resolved, but the assignment of the Austrian specimens as *T. clandestinus* is the most reliable at the current state of knowledge. The habitat of the Austrian location concurs with those of autochthonous populations in Switzerland. Nevertheless, for now it cannot be clearly stated whether the species settled in Vorarlberg directly after the end of the last glaciation or whether the current finding is a result of recent anthropogenic introduction.

Key words: barcoding, species distribution, glacial refugia

Introduction

Vorarlberg, the westernmost federal state of Austria exhibits both west and east alpine elements, not only concerning geological aspects (FRIEBE 2004) but also in biogeographical distribution patterns of several biota (see e.g. the numerous west and east alpine species in the list of REISCHÜTZ 1993). Hence postglacial recolonisation of this area, which was totally covered by ice shields during the last glaciation (VAN HUSEN 1986), took at least partly place from other origins than the rest of the formerly glaciated Austrian Alps (BRANDSTETTER & REISCHÜTZ 1993). The latter were recolonized rather from refugia in south-eastern Europe or even the Alps themselves (Schönswetter et al. 2005). Therefore, an excursion to Vorarlberg took place in June 2016 in the course of the "ABOL Pilot project Mollusca". The Austrian Barcode of Life (ABOL, http://www.abol. ac.at/en/) is an initiative that aims to generate and provide DNA-Barcodes of all species recorded from Austria. Main goals of this specific excursion were to obtain more samples of terrestrial molluscs especially west alpine species reaching Vorarlberg on their eastern border and vice versa. In the course of this excursion two specimens of Hygromiidae were collected, which superficially resembled immature specimens of Urticicola umbrosus (C. Pfeiffer, 1828), but lacked the characteristic shell microsculpture (KERNEY et al. 1983) of this species. A closer investigation of these individuals led to an assignment to Trochulus clandestinus (Hartmann, 1821) and represents a new record of this westerly distributed species of the genus Trochulus Chemnitz, 1786. Trochulus clandestinus is a

polytypic species (FALKNER et al. 2002, 2011) consisting of the nominate subspecies *T. c. clandestinus* distributed in large parts of Switzerland and adjacent areas of France and Germany and the geographically restricted *T. c. putonii* (Clessin, 1874) only distributed in the French Vosges Mountains and adjacent areas of the Rhine valley (Fig. 1). Its relation to the closest congeners *T. caelatus* (Studer, 1820) and *T. montanus* (Studer, 1820) seems to be not unambiguously resolved at the current state of knowledge (PROĆKÓW et al. 2014).

Material and Methods

The sampling site was a mixed forest with a creek called Rimsbach (Figs. 1, 2) and accompanying meadows near Bezau in Vorarlberg at 47.380433°N and 9.920817°E (GeoWGS 84). An exact position and elevation of the sampling site was determined using GPS and recorded together with the habitat and landscape structures as described in DUDA et al. (2014). Animals were drowned in heated water as described by KRUCKENHAUSER et al. (2011) and stored in 80% ethanol. The two individuals of T. clandestinus were registered under the internal accession numbers of the Museum of Natural History Vienna as Mollusca NHMW 109000/AL/00977/7833 and Mollusca NHMW 109000/ AL/00977/7971. DNA was extracted with the DNAeasy blood and tissue kit (Qiagen), following the manufacturer's protocol and the final elution volume of 70 µl. A section of the mt COI was amplified with primers, which were modified from FOLMER et al. (1994) for the ABOL Pilot project Mollusca: LCO1490 ABOL Moll 1 (5'-TCAACAAAY-



Fig. 1. Distribution of *Trochulus clandestinus*. Grey shaded area: hitherto known range of *T. c. clandestinus*; grey striped area: range of *T. c. putonii*; black star: first record from Austria, black dots: localities with sequences of *T. clandestinus* from GenBank, 1 - La Neirigue, 2 - Sensetal, 3 - Chur; Abbreviations: A - Austria, CH - Switzerland, FL - Liechtenstein, F - France, I - Italy. Drawing by Natural History Museum Vienna.



Fig. 2. Sampling site at the Rimsbach near Bezau, Vorarlberg, Austria. Photo by Michael Duda.



Fig. 3. The recorded specimens of *T. clandestinus* in frontal, ventral and dorsal view. A: Mollusca NHMW 109000/AL/00977/7833, B: Mollusca NHMW 109000/AL/00977/7971. Scale bar is 5 mm. The white arrow indicates the blunted keel. Photo by Natural History Museum Vienna.

CATAARGAYATTGG-3') and HCO2198_ABOL_Moll_1 (5'-TAAACTTCTGGRTGACCAAAAAAYCA-3').

The resulting sequence (without primers) was 655 bp in length. The 16S fragment was amplified with the primers 16Sfw 5'-CGCAGTACTCTGACTGTGC-3' (PFENNINGER et al. 2003) and 16S_sch_rev 5'-CG CCGGTCTGAACT-CAGATC-3' (DUDA et al. 2011) and had a length without primers of 359 bp. PCRs were performed on a Master Gradient thermocycler (Eppendorf) in 25 µl with 1 µl template DNA, 0.5 unit Q5 DNA polymerase (BioLaps), 0.5 µM of each primer and 0.2 mM of each dNTP (Roche). Each PCR comprised 35 reaction cycles with an annealing temperature of 55°C (COI) or 50°C (16S). Control reactions were carried out for both DNA extractions and PCR amplifications. PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and analysed by direct sequencing (both directions). Sequencing was performed at LGC Genomics (Berlin, Germany) using the original PCR primers. Sequences are stored in BOLD: ALNHM362-17.COI-5P, ALNHM362-17.16S and ALNHM363-17.COI-5P, ALNHM363-17.16S.

Sequences of this study were compared with sequences of GenBank. For the 16S fragment four individuals from two different localities and for the COI fragment eight individuals from three localities are available for comparison. One of those sequences contained 10 undefined nucleotides (accession number DQ251519.1) and was therefore excluded for comparison.

Both animals were photographed in several different views, their lower genital duct was removed. Shell photographs were taken with a Nikon digital sight D3-Fi1 camera fixed on Wild M420 stereomicroscope. Photos of shells and complete genital tracts were taken at lowest magnification $(5.8\times)$, while penis cross sections were examined at highest magnification $(35\times)$. All photographs were created as extended depth of field images with Nikon software. Geni-

tal morphology and coarse shell morphology of the two Austrian specimens was compared with those of the three Swiss specimens investigated by KRUCKENHAUSER et al. (2014) and DUDA et al. (2014) and drawings of PROĆKÓW (2009).

Results

Morphological and genetic results unambiguously confirmed the identity of these two specimens as west-alpine members of the genus *Trochulus*, most likely *T. clandestinus*. Besides 17 other taxa of land snails were found at the sampling site Rimsbach: *Aegopinella nitens*, *Arianta arbustorum*, *Arion vulgaris*, *Bulgarica cana*, *Cepaea nemoralis*, *Cochlodina laminata*, *Discus rotundatus*, *Ena montana*, *Fruticicola fruticum*, *Helix pomatia*, *Isognomostoma isognomostomos*, *Limax cinereoniger*, *Macrogastra attenuata lineolata*, *Petasina edentula helvetica*, *Trochulus villosus*, *Trochulus hispidus* and *Succinea putris*. Sieving was not performed, therefore small species, which can only be detected by this method, were not recorded.

The two analysed individuals are identical in the sequenced 16S (359 bp) as well as COI (655 bp) fragments. Comparisons with GenBank show high similarity to the published sequences of *T. clandestinus* from PFENNINGER et al. (2005) and KRUCKENHAUSER et al. (2014). The uncorrected p-distances to our sequence in 16S range from 0 to 0.94 %, in COI from 0 to 0.41 % (Tab. 1). Besides the four sequences of PFENNINGER et al. (2005) are situated in Clade 2 of PROĆKÓW et al. (2013). In total, this clade consists of 19 individuals, where additionally six specimens were assigned as *T. caelatus* and nine as *T. montanus*. Shells (Fig. 3) were 9.5 mm (Mollusca NHMW 109000/AL/00977/7833) and 9 mm (Mollusca NHMW 109000/AL/00977/7971) wide and each 4.6 mm high. These measures fall in the lower end of the size range of the species compared to

the values of 9.3-12.3 and 5.6-7.0 provided in literature (KERNEY et al. 1983, PROĆKÓW 2009). This and the fact that both individuals did not have a developed lip and a strong inner lip callus indicate that their shells were perhaps not fully-grown. Both individuals showed five whorls and a prominent blunted keel situated in the upper part of the last whorl in lateral view. The 3rd, 4th and 5th whorl increased quickly in broadness. This fact is mentioned as a relevant descriptive determination trait by some authors (FALKNER 1990, KERNEY et al. 1983, BOSCHI 2011). Also the umbilicus increased rapidly at the last whorl. Although the shells were not fully-grown, the lower genital organs appeared to be adult. The specimens investigated by KRUCKENHAUSER et al. (2014) and DUDA et al. (2014) are similar in shell size as they ranged from 8.9-9.5 mm in width and 4.9-5.4 mm in height. They also showed rapidly increasing whorls, but not this extreme blunted keel.

Coarse genital sections showed a bulky penis and a bursa copulatrix on a comparably short duct (Fig. 4). The penis papilla itself was shown to be flattened laterally, slightly broader in the proximal part, showing a slight concave outline, with the seminal duct ending in a crescent-shape exit situated in proximal direction. The concave outline leads to a remarkable pit in distal direction of the penis (Fig. 5). All these traits are constantly present in new detected Austrian specimens and the three existing specimens investigated in KRUCKENHAUSER et al. (2014) and DUDA et al. (2014) (Figs. 4–6), but also PROĆKÓW (2009) pictured a flattened penis.

Also the penial cross sections showed similarities to previously published data. Above the proximal situated seminal duct a couple of horizontally orientated folds can be recognized. These patterns are also present in sketch drawings of PROĆKÓW (2009) and the specimen pictured in DUDA et al. (2014) (Fig. 6).

Comparisons to the other similar congener show, that these two Austrian specimens definitely do not refer to the *T. hispidus/sericeus* complex, *T. striolatus* and very likely not to *T. caelatus*, as these species show a differing inner structure of the penis. Nevertheless, there is some insecurity for distinguishing it from *T. montanus*, as no detailed inner features are available for this species. For specimens investigated in PROĆKÓW et al. (2014), there were some anatomical features documented.

Discussion

Genetics, detailed anatomical investigations and published distribution data (TURNER et al. 1998, BOSCHI 2011) indicate that these two individuals belong to a clade of *Trochulus* native in the Jura Mountains yet not known from Austria, perhaps most likely *T. clandestinus* (W. Hartmann, 1821). Genetic results indicate, that the snails found at the Rimsbach are very closely related to the Swiss population of *T. clandestinus* investigated by KRUCKENHAUSER et al. (2014) and DUDA et al. (2014) as well as to those from PFENNINGER et al. (2005). Nevertheless, PROĆKÓW et al. (2014) showed a phylogenetic tree with unresolved relations of the three north-west alpine distributed taxa *T. caelatus, T. clandestinus* and *T. montanus* showing these species mixed up in three clades. Their clade 2 was the only

one containing individuals assigned as *T. clandestinus*. If this happened due to hybridization, incomplete lineage sorting or misidentification of species cannot be clarified, as they just used measurements of shells and outer genitalia for a morphological determination. Therefore, the classification of these two Austrian specimens as *T. clandestinus* appears not completely sure at the current state of knowledge, although some points would favour it (see below). For clarifying this contradictions detailed investigations including specimens from the entire distribution area of the three species would be necessary.

In any case, descriptions and measurements of the shell seem not to be reliable traits to separate T. clandestinus from T. caelatus and T. montanus. The prominent not centred keel in the very upper part of the last whorl, which was evident in the currently found specimens, is not pictured in some common determination literature (KERNEY et al. 1983, Boschi 2011). HAUSSER (2005) mentions that the last whorl is slightly keeled and therefore it is a trait to separate T. clandestinus from T. montanus. If this trait really could help separating this species from its congeners T. montanus, T. caelatus and T. striolatus appears doubtful according to Proćków et al. (2014). Their results reconfirm, that shell forms and dimensions are not suitable to separate these taxa. The difficulty to separate congeners by shell dimension has been already shown by other authors (KERNEY et al. 1983, GIUSTI & MANGANELLI 1992, KIRCHNER et al. 2015, RAZKIN et al. 2016).

Nevertheless, two facts favour the identity of our specimens as *T. clandestinus*: (1) The real striking and unambiguous trait is the peculiar laterally flattened penis with the distal pit (Fig. 5), which has not been recorded in this clarity yet. Outer and inner forms of the penis are so unique, that they differ significantly from all other Austrian Hygromiidae. The presence of fully developed genitalia simultaneous with not totally grown peristome traits like lip is nothing new within land pulmonates. Similar findings are documented within other species of the genus, especially *T. hispidus* and *T. striolatus* (DUDA et al. 2014), but also species from other genera like *Arianta arbustorum* (see BAUR (1984) with citations showing these phenomenon also in other families).

A penial section of *T. caelatus* in PROĆKÓW (2009) shows an inner structure completely different from those of *T. clandestinus*, so that an assignment for the newfound snails to *T. caelatus* can be excluded. For the relation to *T. montanus* the case remains complicated as no detailed descriptions or pictures of internal genital structures are published. Also two of the specimens assigned to *T. montanus* in Clade 2 of PROĆKÓW et al. (2014) were collected at type localities of this species, but distribution issues lead to another point favouring the assignment of the Austrian specimens to *T. clandestinus*.

(2) *T. clandestinus* is the one of the three north-west Alpine species (referred as Jura-clade by PFENNINGER et al. 2005) which is far-distributed across Switzerland and still spreading, while *T. montanus* is restricted to the Jura mountains and *T. caelatus* even more restricted to a small part of the Jura Mountains (TURNER et al. 1998, BOSCHI 2011). Besides, the type locality of *T. clandestinus* is situ-



Fig. 4. Lower genitals of *Trochulus clandestinus*, shown on specimen Mollusca NHMW 109000/AL/00977/7833 in slightly contorted original position (left) and as rectified drawing (right). BC – bursa copulatrix, DS – dart sacs, EP – epiphallus, FL – flagellum, HD – hermaphroditic duct, MG – mucus glands, PP – penis papilla, RM – retractor muscle, VD – vas deferens. Scale bar is 5 mm. Photo by Natural History Museum Vienna. Drawing by Michael Duda.



Fig. 5. Penis papilla of *Trochulus clandestinus* in frontal (left) and lateral (right) view, penis sheath removed. LP – lateral pit, SD – outlet of seminal duct. Scale bar is 1 mm. Drawing by Michael Duda.



Fig. 6. Comparison of inner penial structures of *Trochulus clandestinus*. Left: penial cut of Mollusca NHMW 109000/AL/00977/7833, centre: drawing of the same specimen, right: drawing of the specimen pictured in Duda et al. (2014) (COI Accession number KJ 151 365.1). White: cut surface. Grey: rest of penial tissue. Black: deeper folds and ducts. LP – lateral pit, SD – seminal duct, PS – penis sheath. Scale bar is 1 mm. Photo by Natural History Museum Vienna. Drawing by Michael Duda.

Table	1. Inf	ormation	on specimer	is used for	genetic	comparisons.	The	p-distance	between	the A	ustrian	and the	Swiss	specimen	ns is
given.	Cit. =	Citation	: 1: cited in l	PFENNINGER	et al. (2	2005), 2: cited	l in K	RUCKENHAU	JSER et al	. (201-	4).				

Individual Id	Locality	Latitude	Longitude	Cit.	16S GenBank- accession number	p-distance	COI GenBank- accession number	p-distance
CH18_1	Sensetal, Bern, Switzerland	46.8294	7.3219	1	DQ217802.1	0.94	DQ251519.1	n.d.
CH18_5	Sensetal, Bern, Switzerland	46.8294	7.3219	1			DQ251520.1	0
CH3_2	La Neirigue, Fribourg, Switzerland	46.7044	6.9200	1		_	DQ251503.1	0.41
СН3_3	La Neirigue, Fribourg, Switzerland	46.7044	6.9200	1		-	DQ251504.1	0.2
СН3_5	La Neirigue, Fribourg, Switzerland	46.7044	6.9200	1		_	DQ251505.1	0.41
1381_199	Chur, Graubünden, Switzerland	46.8123	9.5325	2	KJ151586.1	0.56	KJ151365.1	0
1382_199	Chur, Graubünden, Switzerland	46.8123	9.5325	2	KJ151587.1	0.56	KJ151366.1	0
1383_199	Chur, Graubünden, Switzerland	46.8123	9.5325	2	KJ151588.1	0	KJ151367.1	0.31
					16S BOLD ID		COI BOLD ID	
Mollusca NHMW 109000/ AL/00977/ 7833	Bezau, Bregenzerwald, Austria	47.3804	9.9208	this study	ALNHM363- 17.16S		ALNHM363- 17.COI-5P	
Mollusca NHMW 109000/ AL/00977/ 7971	Bezau, Bregenzerwald, Austria	47.3804	9.9208	this study	ALNHM362- 17.16S		ALNHM362- 17.COI-5P	

ated near Zürich outside of the Jura Mountains in contrast to the other two species. Also the drawing of genitals in PROĆKÓW (2009), which shows accordance to the currently found specimens and the ones pictured in DUDA et al. (2014) originate from a specimen from outside the Jura Mountains. Also habitat preferences and spreading behaviour support the assumption that the new discovered population belongs to *T. c. clandestinus*. TURNER et al. (1993) and BOSCHI (2011) mention that "riverside shrubberies" are primary habitat in Switzerland, but *T. c. clandestinus* is also able to settle in cultivated areas. This ability to spread in anthropogenous habitats led to an enlargement of its original distribution area during the last 150 years (TURNER et al. 1993, BOSCHI 2011, WIESE 2016).

In the case that the newly found Austrian specimens really represent *T. clandestinus* and the currently used nomenclature stays stable, they can be very likely assigned to the nominate subspecies *T. c. clandestinus*. This is supported also by another fact: *T. c. clandestinus* was originally only distributed in the very northern parts of the Swiss Alps, which remained ice-free during the last glaciation and extended its distribution range to the west and north during the last 150 years and is still spreading (TURNER et al. 1998, FALKNER et al. 2011, BOSCHI 2011). Other populations of the nominate subspecies are also situated close to the state borders in Switzerland and Liechtenstein (Fig. 1). In opposite to that, the second subspecies, *T. c. putonii* is geographically separated and strongly declining (FALKNER et al. 2011) which makes its occurrence in Austria very unlikely. Nevertheless, *T. c. putonii* should be also subjected to a thorough revision, as only coarse descriptions for this taxon like "smaller shell size" and "more convex last whorl" (FALKNER et al. 2002) are available, but this is also beyond the scope of the current study.

These facts might allow two interpretations of the origin of the Austrian *T. clandestinus*. On one hand, the sampling site Rimsbach near Bezau is characterized by a creek with accompanying high perennial herbs and a damp deciduous forest (Fig. 1) and thus resembles the natural habitat of the species in Switzerland. Therefore, a colonization of Vorarlberg after the last glacial period around 10,000 years BP seems possible. On the other side, there had been already some efforts to find this species in Vorarlberg in the 1990s (remark in TURNER et al. 1998) but with no positive results. Besides, also a quite recent anthropogenic introduction to Austria should be considered. The high genetic similarity of all investigated specimens of *T. clandestinus* is in contrast to other species of the genus *Trochulus* (KRUCK-ENHAUSER et al. 2014) and indicates a quick expansion to the recent distribution area that might have occurred under human influence; however colonization right after the end of last glaciation can still not be ruled out.

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