



universität
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MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Distribution and DNA barcoding of hydrobioids
(Gastropoda) from the Kalkalpen National Park (Austria)“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of
Master of Science (MSc)

Wien, 2021 / Vienna, 2021

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on
the student record sheet:

UA 066 831

Studienrichtung lt. Studienblatt /
degree programme as it appears on
the student record sheet:

Masterstudium Zoologie UG2002

Betreut von / Supervisor:

Mag. Dr. Luise Kruckenhauser, Privatdoz.

Acknowledgements

My special thanks go to Luise Kruckenhauser, who supervised my work and supported me at all times with her professional knowledge and also with her personal support. Thank you for your time, your patience and for giving me a push when I needed it. I would also like to thank the Natural History Museum and especially the Central Research Laboratories department with its director Elisabeth Haring for receiving the opportunity to write my master's thesis at a place of knowledge and research. Many thanks also to Michael Duda, who not only introduced me to the world of snails but was also always there to help me and with whom I've always enjoyed working. I would like to thank the Kalkalpen National Park and especially Erich Weigand for the exciting project and the extensive collecting work in advance. Thanks also to Anita Eschner for her constructive and friendly advice in writing, for transferring the samples to the museum collection and for taking the time, even if she had none. I would like to thank Julia Schindelar for her time and care she took in introducing me to the lab work and for her patience when I asked some of the questions several times. Furthermore, my thanks go to Martin Haase for his confirmation of the snails' identification. I would also like to thank ABOL – the Austrian barcode of life Initiative for sharing their data and in the course of this all the collectors, especially Alexander Reischütz, Otto Moog and Alexander Mrkvicka.

In addition, I would like to thank the whole Lab Crew for their support, for their help and especially for the fun we had together.

Finally, for funding I would like to thank The Austrian Research Promotion Agency (FFG) (FEMtech internships for female students) and The Federal Ministry of Agriculture, Regions and Tourism within the scope of Austrian Rural Development Programme 2014-2020.

Outside of my work environment, I would of course like to thank my family and friends who always stand by me and never let me forget what is really important in life.

Abstract

The Kalkalpen National Park is situated in Upper Austria and contains more than 800 springs. The international importance of this park is, seen from the perspective of nature conservation directives, highly significant (European nature reserve Natura 2000, recognized wetland of the Ramsar convention). In the current study the hydrobioid fauna ('spring snails') of the Kalkalpen National Park was evaluated. These tiny snails are difficult to determine, however, their investigation is especially desirable, as several species are threatened and as they are important for water quality assessment. The snails of 39 selected springs were examined with classical methods (shell morphology and in some cases genital anatomy) and with DNA barcodes. Sequences of a approx. 670 bp fragment of the mitochondrial COI gene were generated for 107 specimens and could be compared with already existing DNA sequences. It could be shown that in some springs of the Kalkalpen National Park rare hydrobioids, which are due to their endemic status particularly important to protect, occur in varying frequencies. The species *Bythinella conica*, *Hauffenia kerschneri*, *Hauffenia wienerwaldensis* and *Belgrandiella aulaei* could be clearly identified by morphological and molecular methods. For *Bythiospeum nocki*, despite the ambitious collecting effort, only empty shells were found. The data obtained can contribute to the assessment of the taxonomic classification of the species studied. The present study gives a good baseline for further monitoring of the hydrobioids in the Kalkalpen National Park or similar environments, which is important to evaluate current and decide on future protection measures for this group.

Zusammenfassung

Der in Oberösterreich gelegene Nationalpark Kalkalpen umfasst mehr als 800 Quellen. Dieser Park hat aus Sicht der Naturschutzrichtlinien eine sehr hohe internationale Bedeutung (Europaschutzgebiet Natura 2000, anerkanntes Feuchtgebiet der Ramsar-Konvention). In der vorliegenden Studie wurde die Hydrobioden-Fauna ("Quellschnecken") des Nationalparks Kalkalpen untersucht. Diese winzigen Schnecken sind schwer zu bestimmen, ihre Untersuchung ist jedoch besonders wünschenswert, da mehrere Arten bedroht und für die Beurteilung der Wasserqualität wichtig sind. Die Schnecken von 39 ausgewählten Quellen wurden mit klassischen Methoden (Schalenmorphologie und in wenigen Fällen Genitalanatomie) und mittels DNA-Barcoding untersucht. Sequenzen eines ca. 670 bp-Fragments des mitochondrialen COI-Gens wurden für 107 Exemplare erstellt und konnten mit bereits vorhandenen DNA-Sequenzen verglichen werden. Es konnte gezeigt werden, dass in einigen Quellen des Nationalparks Kalkalpen seltene Hydrobioide, die aufgrund ihres Status als Endemiten besonders schützenswert sind, in unterschiedlicher Häufigkeit vorkommen. Die Arten *Bythinella conica*, *Hauffenia kerschneri*, *Hauffenia wienerwaldensis* und *Belgrandiella aulaei* konnten eindeutig morphologisch und genetisch identifiziert werden. Von *Bythiospeum nocki* wurden trotz des intensiven Sammelaufwands nur leere Schalen gefunden. Die erhobenen Daten können zur taxonomischen Beurteilung der untersuchten Arten beitragen. Die vorliegende Studie bietet eine gute Grundlage für das weitere Monitoring der Hydrobioden im Nationalpark Kalkalpen oder in ähnlichen Umgebungen, was von hoher Wichtigkeit ist, um aktuelle und zukünftige Schutzmaßnahmen für diese Gruppe zu evaluieren.

Content

Acknowledgements	3
Abstract	4
Zusammenfassung.....	5
1. Introduction.....	9
1.1 General Background	9
1.1.1 Hydrobioids.....	9
1.1.2 Hydrobioids in Austria	11
1.1.3 Kalkalpen National Park.....	12
1.1.4 DNA Barcoding and the Barcode of Life Data System (BOLD).....	13
1.1.5 ABOL-Initiative	13
1.2 Initial situation.....	14
1.2.1 Hydrobioids in the Kalkalpen National Park.....	14
1.2.2 Endemism, endangerment and conservation status within the hydrobioids	16
1.2.3 Genetic studies on hydrobioids: COI and other marker genes	17
1.3 Aims	17
2. Material & Methods	19
2.1 Sampling and specimens processing	19
2.2 Morphology and anatomical examination.....	20
2.3 DNA extraction and COI amplification	22
2.4 Data analyses.....	23
3. Results	25
3.1 Collecting success and photo documentation	25
3.2 Morphological identification of species.....	27
3.3 DNA barcoding success.....	31
3.4 Haplotypes of the Kalkalpen National Park	32
3.5 Sequence comparison with the ABOL Mollusca project	32
3.6 BOLD analysis and Barcode Index Numbers (BINs).....	36
3.7 Distribution of Hydrobioids in the Kalkalpen National Park	38
4. Discussion	42
4.1 Discussion of methods.....	42
4.1.1 Sampling method.....	42
4.1.2 Laboratory Work.....	42
4.1.3 BOLD analysis.....	43
4.2 Species determination and delimitation	43
4.2.1 Assessment of the species <i>Belgrandiella aulaei</i>	45
4.2.2 Assessment of the species <i>Bythinella conica</i>	47
4.2.3 Assessment of the species <i>Bythiospeum nocki</i>	48
4.2.4 Assessment of the species <i>Hauffenia kerschneri</i> and <i>Hauffenia wienerwaldensis</i>	49
4.3 Species distribution inside the Kalkalpen National Park.....	50
4.4 Endangerment and Conservation	52
5. Conclusio	53
References.....	55
Supporting Information.....	60

1. Introduction

Biodiversity - the variety of life on earth - encompasses all living organisms and their diverseness. This includes the diversity of species, the diversity within species, and the diversity of communities of species. In recent years, a high loss of biodiversity has been recorded, partly caused by humans. Knowledge about the diversity of nature is the key prerequisite for developing strategies to protect it.

Small-scale monitoring of biodiversity includes examining the abundance and distribution of a group of organisms to detect long-term changes. National parks and other protected areas are subject to a reporting obligation on the status of their protected areas and protection measures. To be able to show changes in biodiversity and biodiversity loss, the status quo must be recorded regularly. This process is usually very labour-intensive and only possible with existing taxonomic expertise.

In the current study a monitoring of a selected group of animals, namely the hydrobioids (spring snails) in the Austrian Kalkalpen National Park and its surroundings was performed. Classical monitoring reaches its limits with the small spring snails that are morphologically difficult to determine, which is why the DNA barcoding tool is used here.

1.1 General Background

1.1.1 Hydrobioids



Figure 1: Two specimens of the species *Bythinella conica*; photo and copyright by Alexander Mrkvicka

A large number of different genera of very small spring- and groundwater- snails have been classified in biology under the family Hydrobiidae. Due to taxonomic revisions, these genera are now partly assigned to different families. The functional term hydrobioids allows a common naming of these genera and is therefore explained and used in the following.

Hydrobioid is a non-taxonomic, functional term for the totality of hydrobiid (Hydrobiidae Stimpson, 1865) and hydrobiid-like taxa, first defined by Davis (1979) and later reused by Kabat and Hershler, 1993, who subjected the family Hydrobiidae s.l. Troschel, 1857 to a review and Wilke et al. (2013), who have studied the phylogenetic relationships of hydrobioids. Davis lists 14 points to define the group of hydrobioids, which include the general characteristics of foot, operculum, genitalia, fecal pellets, and pigmentation. In the present study, the term is used primarily for the former family Hydrobiidae s.l., which included all the investigated species in this study. The use of this term is necessary, because the monophyly of the family Hydrobiidae s.l. was clearly rejected by Wilke et al., (2001, 2013). The suggestion of Wilke et al. (2013) to no longer use the designations Hydrobiidae s.s. and s.l. is followed (except to clarify to which group the literature refers) and instead the term hydrobioids is used for taxa similar to Hydrobiidae (Hydrobiidae after Troschel, 1857). In this study, the families Bythinellidae Locard, 1893, Hydrobiidae Stimpson, 1865 and Moitessieriidae Bourguignat, 1863 are considered in particular.

All hydrobioids are very small, 0.5 to 8 (maximum 15) millimetres in size (Miller et al., 2018), gonochoristic freshwater gastropods. Their vernacular name ‘spring snails’ is due to the fact that most of the stygobiont snails live in springs, but also caves and interstitial habitats (Falniowski, 2018). Other habitats of hydrobioids are streams and rivers, lakes, groundwater systems, estuarine marshes and mudflats (Strong et al., 2008 (Hydrobiidae s.s.)). They have a low ability for dispersal and a limited distribution range (Miller et al., 2018 (Hydrobiidae s.s.); Strong et al., 2008). Sympatric occurrence of different species of the same genus is rare (Glöer, 2002 (Hydrobiidae s.l.); Wilke et al., 2010).

Hydrobioids include the most genera within the freshwater gastropods (Glöer, 2002 (Hydrobiidae s.l.)). Over 1000 species are described (Falniowski, 2018), Strong et al., 2008 estimate the possible number to be in the order of 8000. The classification of hydrobioids is largely based on shell morphology and distribution (Falniowski, 2018). Although hydrobioids account for a large number of new species descriptions (Strong et al., 2008), the description of a new species solely on the basis of a new range can lead to an overestimation of the actual number of species (see Richling et al., 2016).

Several taxa of hydrobioids are morphologically and anatomically highly variable (Wilke et al., 2013). Hence, both, delimiting species and assigning individuals to an existing species, is very difficult only by morphological methods. Even though Boeters (1999) gives well developed instructions about the preparations of small Prosobranchia, there are still only few robust anatomical characters, which could

be used for determination. Reasons for this are, that, because of their small size, hydrobioids have a reduced morphology and that convergence in anatomical features is common in Rissooidea (Kabat and Hershler, 1993 (Hydrobiidae s.l.)). Wilke et al. (2001) note in addition, that intraspecific variation of anatomical characters is very high in hydrobioids (Hydrobiidae s.l.). Delicado and Ramos (2012) pointed out, that for the delimitation of hydrobioids molecular data would be useful to support morphological analyses (Delicado and Ramos, 2012 (Hydrobiidae s.s.)). Falniowski (2018) also confirms the need for molecular data in this context.

Among the hydrobioids many species are endemic (Strong et al., 2008). Miller et al. (2018)) found 83% of their 906 studied hydrobioid species (Hydrobiidae s.s.) as endemics. Because of their restricted distribution they are highly endangered by habitat loss (Miller et al., 2018 (Hydrobiidae s.s.)). One destruction event may be enough to wipe out the only known population of a species (Strong et al., 2008) and thus can lead to extinction. Of the 1117 species of hydrobioids (here the old sensu lato definition of Hydrobiidae is still used) listed on The IUCN Red List of Threatened Species (status March 2020), 31 are extinct and 536 (ca. 48%) are at least vulnerable. Miller et al. (2018) predict that there will be a higher risk for hydrobioids (Hydrobiidae s.s.) in the future, because of global climate change and the resulting destruction of ecosystems.

1.1.2 Hydrobioids in Austria

Reischütz and Reischütz, 2007 listed 42 hydrobioid species of 9 genera for Austria (currently belonging to five different families). 35 of them were classified as endemics and three as subendemics. The authors categorise one species as not evaluated, two as data deficient, one as least concern, four as near threatened, one as vulnerable, three as endangered, 28 as critically endangered and four as extinct. A full list of all Austrian hydrobioid species by Reischütz and Reischütz (2007) can be found in Supporting Table 1. Excluding the only invasive hydrobioid species *Potamopyrgus antipodarum* Gray, 1843 (Glöer, 2002 (Hydrobiidae s.l.)), all native hydrobioids require uncontaminated to very low contaminated waters (Nesemann and Reischütz, 2002 (Hydrobiidae s.l.)). The presence, decline or even absence of these spring inhabitants allows conclusions to be drawn about the quality of the water, which also makes them ideal bioindicators or indicator species (Nesemann and Reischütz, 2002; Zulka, 2014 (Hydrobiidae s.l.)). Reischütz and Reischütz (2009) criticize the way hydrobioids (Hydrobiidae s.l.) are protected in Austria. One example the authors give, is that the protection of all hydrobioids in general (as it is the case in Lower Austria), also includes the invasive species *P. antipodarum* (a harmful organism). Another is that habitats (i.e. springs) continue to be destroyed, among others for drinking water production.

1.1.3 Kalkalpen National Park

The Kalkalpen National Park, hereinafter abbreviated to Kalkalpen NP, is situated in Upper Austria and comprises approx. 209 km². It is of utmost importance from a nature conservation perspective. Established in 1997, the area has been internationally recognised as a national park (according to IUCN category II) since 1998. Since 2004, the NP is a recognized wetland of the Ramsar Conservation and also since 2004 part of the European nature reserve Natura 2000. In addition, parts of the Kalkalpen NP (beech forests, which together make up about a quarter of the total area of the park) have been declared to one of Austria's first UNESCO World Natural Heritage Site (Nationalpark O.ö. Kalkalpen Ges.m.b.H, 2018).

The Kalkalpen NP comprises more than 800 springs (see Figure 2). These springs reflect the characteristics of their catchment area and indicate environmental changes, human interventions, and disturbances of the catchment area. The abundance of springs in the park is typical for the karst landscape and gives rise to a variety of spring forms. They can provide a suitable habitat for highly specialised species. The national park staff has been researching the springs since its beginnings and carries out detailed monitoring of some of them, evaluating physical, chemical and microbiological parameters (Stadler, 2017).

Springs of the Kalkalpen National Park

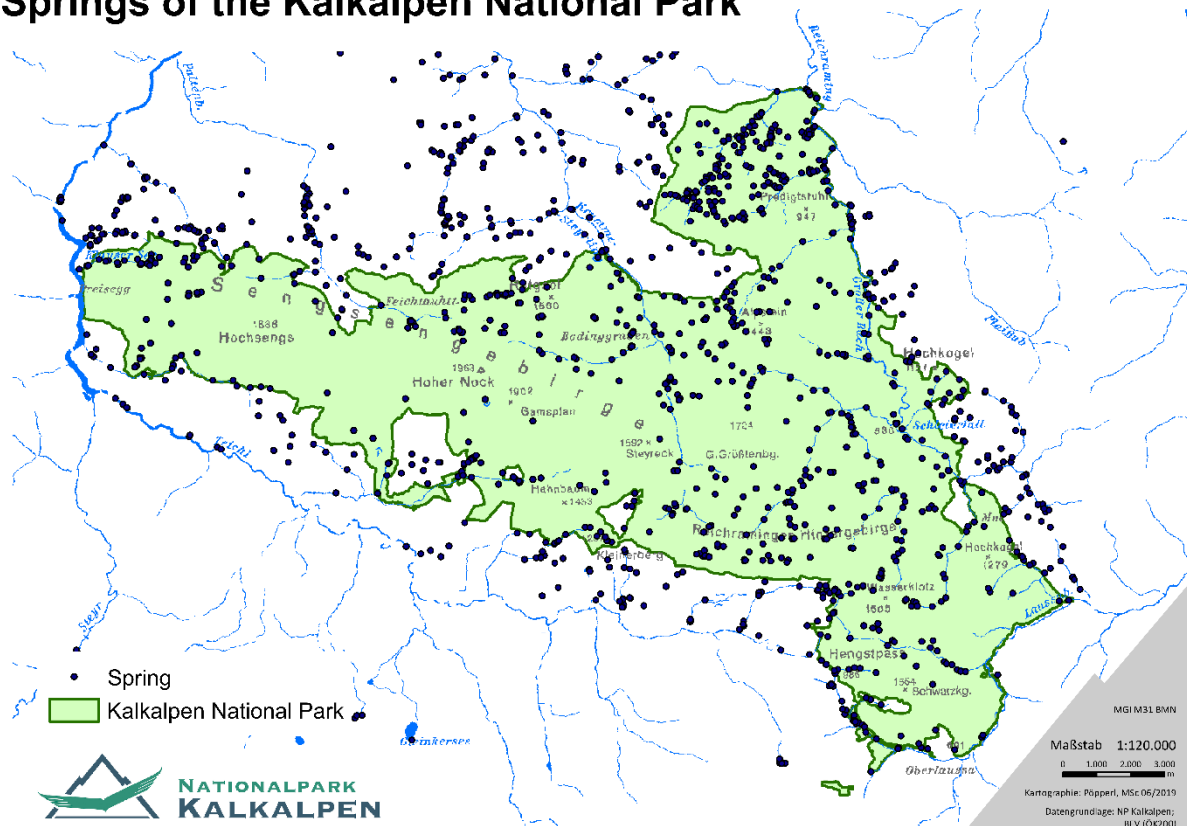


Figure 2: Springs of the Kalkalpen National Park and its surroundings; copyright by Management of the Kalkalpen National Park

The Kalkalpen NP is of particular importance for biodiversity in Austria, in accordance with its function as a "hot spot" for endemics and Red List species, which extends beyond the region (Steger, 2012). Steger (2012) lists eight endemics and one subendemic for the national park area as well as 15 Red List mollusc taxa. The high number of endemics and other gastropods worth being protected (especially in the area of springs and alpine regions of the park (see Steger, 2012)) is of great significance for Austria.

1.1.4 DNA Barcoding and the Barcode of Life Data System (BOLD)

In the year 2003 Hebert et al. introduced the term, DNA barcoding, for using molecular tools for animal species identification. A major argument for DNA barcoding is the great diversity of life and the collapsing taxonomic expertise. Where morphological species identification comes to its limits, for example with cryptic taxa, morphological variation, phenotypic plasticity, different life stages, or small samples of organisms, molecular methods should help. The DNA barcoding method is about comparing a specific sequence of a gene segment between different taxa or individuals. The mitochondrial gene cytochrome c oxidase subunit 1 (COI) turned out as a good marker and now an approximately 650 bp long fragment of the gene sequence is used as a standard for DNA barcoding in animals. DNA barcodes can also be used as a tool for species delimitation. A way to investigate molecular species delimitation is the use of thresholds. These should distinguish between intra- and interspecific variation (Hebert et al., 2003). However, the occurrence of a clear so-called "barcoding gap" between these two is viewed critically (Wiemers and Fiedler, 2007).

The Barcode of Life Data System (BOLD) is a worldwide publicly accessible database in which DNA barcoding data can be provided. In addition to the DNA sequences and the corresponding species names, metadata such as collecting information, determination information, electropherograms of the sequences and images can be uploaded. Apart from a database, BOLD has analytic features, like the Barcode Index Number (BIN) System, where similar sequences are grouped into clusters (operational taxonomic units) (Ratnasingham and Hebert, 2007, 2013).

1.1.5 ABOL-Initiative

ABOL – Austrian Barcode of Life (www.abol.ac.at) is an initiative to record the Austrian biodiversity of animals, plants and fungi in an integrative approach that includes DNA barcoding as a standardized method (Szucsich, 2015). The initiative represents a national network of institutions and experts, which seeks to meet the growing demand for biodiversity-specific expertise (environmental impact assessments, monitoring obligations, climate change), that is facing the growing shortage of taxonomic specialists. Genetic identification methods, in particular DNA barcoding, offer a reproducible, cost-

effective and efficient species identification tool that can complement classical anatomical and morphological methods (Kruckenhauser et al., 2019). The DNA barcoding of Austrian molluscs is one project of the Austrian Barcode of Life initiative (www.abol.ac.at/project/mollusken/), which is conducted since 2014 at the Natural History Museum Vienna (NHMW). For this purpose, freshly collected material on the one hand and older museum material on the other, is used. The DNA barcodes, which were generated with a well-established workflow, were uploaded to the BOLD database (<http://v4.boldsystems.org/>) and will be made accessible to the public (Kruckenhauser et al., 2019). Until now (outside the present project) approx. 730 DNA barcodes of approx. 230 different molluscs species could be generated for the ABOL Mollusca project (Status June 2021), of which 81 DNA barcodes of 17 species belong to the hydrobioids.

1.2 Initial situation

1.2.1 Hydrobioids in the Kalkalpen National Park

The state of knowledge on the occurrence of hydrobioids in the Kalkalpen NP is very incomplete. First surveys yielded two new species for the area, *Belgrandiella aulaei* Haase, Weigand & Haseke, 2000 and *Bythiospeum nocki* Haase, Weigand & Haseke, 2000 (Haase et al., 2000). These newly discovered species were studied morphologically and anatomically, respectively. In the following years, only a few further studies were carried out on the spring-dwelling snails of the national park. In total, four species or species complexes from the genera *Bythiospeum* Bourguignat, 1882, *Belgrandiella* A. J. Wagner, 1928, *Bythinella* Moquin-Tandon, 1856 and *Hauffenia* Pollonera, 1898 were detected (Aescht and Bisenberger, 2011; Haseke and Weigand, 2000; Steger, 2012; Weigand, 2012, 2016). In addition to the two newly described species, the occurrence of the species *Bythinella austriaca* (Frauenfeld, 1856), *Bythinella conica* Clessin, 1910 and *Hauffenia kerschneri* (S. Zimmermann, 1930) (however, two morphotypes could be identified for the genus *Hauffenia*) is discussed (Steger, 2012; Weigand, 2012). In the final report of a study on the mollusc fauna of the Kalkalpen NP, it was specifically suggested that the hydrobioids of the area should be subjected to a thorough inventory and genetical investigation (Steger, 2012). In the initial situation for the present survey of the hydrobioids of the Kalkalpen NP, it was assumed that a high diversity of spring-dwelling snails can be found in this area, which is characterised by the numerous and less dynamic springs of the Reichraminger Hintergebirge (Stadler, 2017). Some representatives of the previously listed genera in the Kalkalpen NP could not be clearly identified to species level by morphological-anatomical studies due to partly vague descriptions of characteristics, which refer to minor shell-morphological and anatomical differences (partly also intraspecific variation).

The suspected species are described in short monographs. Parts of the descriptions have been taken from German and translated to the best of knowledge.

Belgrandiella aulaei (Fam. Hydrobiidae) was described in the year 2000 by Haase, Weigand & Haseke. The type locality is a spring system, called Rinnende Mauer in the northern surroundings of the Kalkalpen NP. The species was found in the lower springs and their common discharge. So far it is only known from one other spring not far from the locus typicus. The collected individuals were examined morphologically and anatomically concerning shell, operculum, radula, non-genital and genital anatomy. *B. aulaei* has a pupiform shell (mean high between 1.34 and 1.43 mm) with shallow sutures and up to 3.75 whorls and an orange operculum. It is characterized by the position of the receptaculum seminis and a combination of some special forms and positions of anatomical characters. (Haase et al., 2000). This endemic *Belgrandiella* species is critically endangered (Reischütz and Reischütz, 2007). The main threats to these animals are amongst other things: Lowering of the groundwater, destruction of biotopes and water pollution (Reischütz and Reischütz, 2009).

Bythinella austriaca (Fam. Bythinellidae) was described 1856 by Frauenfeld based on shell morphology. As locus typicus cold springs in Dornbach next to Vienna (“in kalten Quellen von Dornbach nächst Wien”) was named. Frauenfeld first assigned the species to the genus *Paludinella* Pfeiffer, 1841 (Frauenfeld, 1856). Geyer amended the classification to the genus *Bythinella* in 1927 (Geyer, 1927). According to The IUCN Red List of Threatened Species (status March 2020) *B. austriaca* is distributed in Austria, Czechia, Germany, Hungary, Poland and Slovakia. The species is near threatened (Reischütz and Reischütz, 2007).

Bythinella conica (Fam. Bythinellidae) was described in the year 1910 on the basis of shell morphology. The type locality is Alzauswurf near [“bei”] Burgkirchen. In the following years it is often listed as a subspecies of *B. austriaca* (for examples see Glöer, 2002; Reischütz and Reischütz, 2007, 2009). In a detailed investigation in 2012, Boeters and Knebelsberger did not find any clear differences in morphology and anatomy of *B. austriaca* and *B. conica* but did find a clear molecular delimitation between the two species. The endemic status of this species is not yet fully resolved (a distribution in Austria and Germany is confirmed) (Boeters and Knebelsberger, 2012). Reischütz and Reischütz (2007) list the species as subendemic and critically endangered. This assessment was based on the knowledge at the time that the species only occurs in the lower Inntal, where it is actually threatened. Later studies (Boeters and Knebelsberger, 2012; Ternus et al., 2019; own studies) have shown that *B. conica* is more widespread in Austria. The endangerment status must therefore be re-evaluated.

Bythiospeum nocki (Fam. Moitessieriidae) was described in the year 2000 together with *B. aulaei* by Haase et al. in the surroundings of the Kalkalpen NP. The type locality is the spring Reutersteinquelle, located in the northern surroundings of the Kalkalpen NP. Additional occurrence is known from two

other springs in the sector (Haase et al., 2000). Their habitat is subterranean (Reischütz and Reischütz, 2009). Unlike *B. aulaei*, *B. nocki* was described only by shell morphology. It is characterized by its small size (mean shell high 1.33 mm), a short spire and a detached peristome. It has a turritiform shell with up to four whorls (Haase et al., 2000). *B. nocki* is an Austrian endemic and critically endangered (Reischütz and Reischütz, 2007). For main threats see *B. aulaei* (Reischütz and Reischütz, 2009).

Hauffenia kerschneri (Fam. Hydrobiidae) was described 1930 as *Horatia erythropomatia kerschneri* by Zimmermann based on shell morphology (Zimmermann, 1930). 1988 this species was assigned to the genus *Hauffenia* by Reischütz (Haase, 1992a). Its locus typicus are the spring drains of the water pipeline [„Quellkanälen der Wasserleitung“] in Weyer an der Enns in Upper Austria (Zimmermann, 1930). *H. kerschneri* is listed as an Austrian endemic and critically endangered (Reischütz and Reischütz, 2007).

1.2.2 Endemism, endangerment and conservation status within the hydrobioids

In general, there is a high number of endemics among native snails (Reischütz and Reischütz, 2007, 2009). In Austria, especially the northern and southern Eastern Alps are a hot spot of endemic invertebrates and vascular plants (Rabitsch and Essl, 2009). The reasons for this are the special biogeographical conditions, which are linked, among other things, to the history of the ice ages. During the ice ages, many areas of the Eastern Alps were glaciated, but especially in the northeastern and southeastern parts the ice sheet was never completely closed. Mountain ranges with several altitudinal levels, vertically continuous areas such as rock and debris areas, as well as groundwater systems offer a potential basis for the formation of endemics. Local survival under adverse climatic conditions is given by the possibility to migrate upslope during warm phases and again downhill when it cools down. The groundwater systems of the unglaciated mountain ranges in the Eastern Alps also offered long-term stable temperature conditions. Accordingly, numerous endemics are found in hydrobioids (35 of the 42 species currently documented for Austria, see chapter 1.2). With its complex groundwater system (Stadler, 2017) and the numerous unobstructed springs, the Kalkalpen NP is of supra-regional importance for this group of animals. Especially the groundwater snails of the genera *Hauffenia* and *Bythiospeum* living in the crevice system of the karst are still insufficiently researched and highly threatened by human impacts or lowering of the groundwater level.

In his prioritisation of Austrian animal species and habitats for nature conservation measures, Zulka, (2014) lists 48 mollusc taxa in the highest prioritisation category for Austria. A large proportion of these are spring- and cave-dwelling snails, as these "can easily be wiped out by accidental interventions in Austria; in the case of spring snails, for example, pollution of the spring is sufficient for this" (Zulka, 2014). Zulka (2014) therefore recommends, in close connection with the biodiversity concept

according to Wilson et al. (1988), to give endangered species a high priority in nature conservation. Accordingly, all measures regulated by national parks and nature conservation laws, as well as other official regulations, such as research and monitoring, protection and management plans, must be adhered to and implemented.

1.2.3 Genetic studies on hydrobioids: COI and other marker genes

Outside the present study 81 DNA barcodes of 17 hydrobioid species could have been barcoded within the ABOL Mollusca Project, including the genera *Belgrandiella*, *Bythinella*, *Bythiospeum*, *Graziana* Radoman, 1975, *Hauffenia*, *Potamopyrgus*, *Iglica* A. J. Wagner, 1928 and *Lithoglyphus* C. Pfeiffer, 1828 (Status June 2021). There are an additional of 2157 DNA barcodes of these genera on BOLD (Status August 2021). Even if some studies just have sequenced a part of the standard DNA barcoding region (for example see Grego et al., 2020; Hofman et al., 2018; Szarowska et al., 2016; Wilke et al., 2013), the COI gene fragment is frequently used for genetic analyses in the hydrobioid group and also provides the most reference data. Wilke et al. investigated the monophyly of hydrobioids and their phylogenetic relationships in 2001. In their study they chose a combination of the phylogenetic markers COI and a fragment of the nuclear 18S gene and found that both fragments show good performance for this purpose. The COI gene fragment has a consistent performance at the genus and family level, while the 18S gene fragment has a good phylogenetic informative value on and above the family level (Wilke et al., 2001). In the following two decades several phylogenetic analyses were done on hydrobioids using COI (for some examples see Benke et al., 2009; Boeters and Knebelsberger, 2012; Delicado, 2018; Delicado and Ramos, 2012; Falniowski et al., 2012; Haase et al., 2007; Richling et al., 2016; Šteffek et al., 2011) or a combination of COI with one or more other markers (for some examples see Bichain et al., 2007; Delicado et al., 2018, 2019; Hofman et al., 2018; Szarowska et al., 2016; Wilke et al., 2013). Richling et al., called COI “a suitable marker for first phylogenetic reconstructions” when studying the genus *Bythiospeum* in Europe in 2016. Wilke et al., who investigated the phylogenetic relationships of hydrobioids in 2013, notes, however, that a “combination of ‘standard’ gastropod genes is very useful for phylogenetic studies targeting family-groups or lower risssoidean taxa”. Delicado (2018), investigated the genus *Sadleriana* Clessin, 1890, notes that the COI fragment “provides sufficient resolution to detect intra- and interspecific variation in springsnails”.

1.3 Aims

The main aim of this study is a detailed survey of the hydrobioid taxa in the springs of the Kalkalpen NP. This is to be achieved by morphological determination, photographic documentation and the

creation of DNA barcodes from hydrobioid snails from more than 30 springs. Above all, endemic species that require special protection should be identified. Furthermore, the generated DNA barcodes are to be compared with existing reference data (e.g. from ABOL and BOLD). In addition, reference DNA barcodes are to be created from newly acquired genetic data. This study will evaluate not only the status quo of the hydrobioids of the Kalkalpen NP but will also serve as a model study and facilitate future monitoring of hydrobioids in the Kalkalpen NP and its surroundings.

2. Material & Methods

2.1 Sampling and specimens processing

The samples were collected from 39 springs of the Kalkalpen NP and its surroundings. The majority of samples were collected by Dr. Erich Weigand from the Nationalpark O.ö. Kalkalpen Ges.m.b.H between October 2018 and April 2020. Different sampling strategies were followed: Collecting by hand, using a fine sieve, scooping with a small container or using a net. In Supporting Table 2 the localities, from which samples were processed during this study, including their abbreviation and additional information on the sites (location, type of spring, drainage direction), are listed. Most of the springs examined were sampled once, in seven cases two collecting events were evaluated. In most cases the collecting was not further than 15 meters from the spring outlet. All in all, the distance varies between 0 to 300 meters.

Additional 81 collected samples, that were not examined in more detail in the context of the present study, have also been included in the mollusc collection of the NHMW and are available for further processing (Acqu.Nr. 2019.V.).

First examinations included the sorting of samples, which were collected by sieving, scooping or by a net. These samples were delivered frozen in volumes between 100 and 500 ml and were then thawed and preserved in 80% ethanol before processing. The specimens were separated from non-organic material and non-mollusc animals and determined at the genus-level by shell morphology (following Glöer, 2002) with a WILD M420 macroscope of Leica Microsystems. Figure 3 shows an example of a sample, that had to be sorted. All specimens from one genus and one collecting event were pooled together and stored in 80% ethanol. Empty shells that were found were stored dry separately. Individuals collected directly by hand could be used without further preparation.

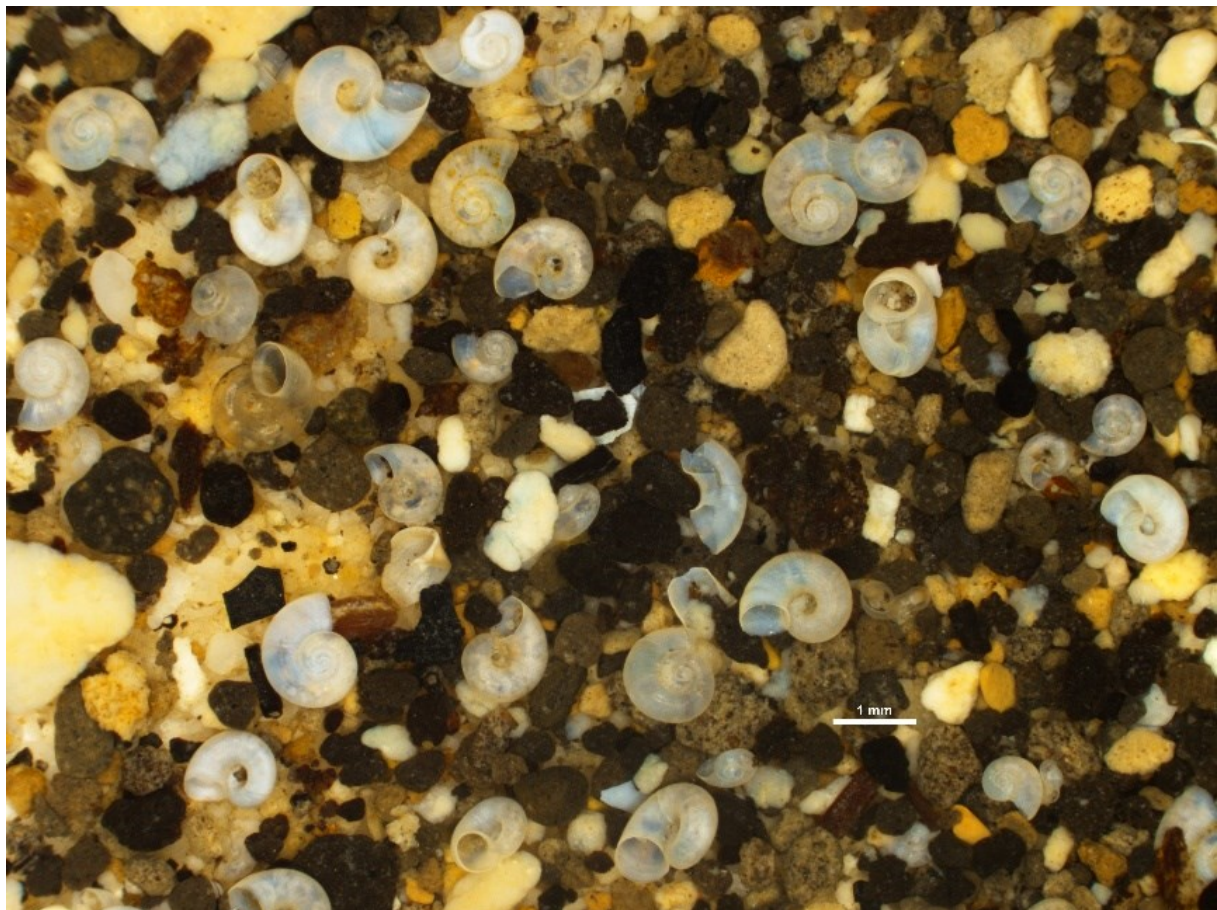


Figure 3: Scooping sample of the spring Rettenbachhöhle including shells of the genus *Hauffenia*

Applying this procedure 58 samples (from 39 different localities) of hydrobioid species were obtained. The samples are listed in Supporting Table 3. Specimens which were selected for molecular analyses and empty shells of the genus *Bythiospeum* were photo documented with a Nikon SMZ25 stereo microscope that was equipped with a Nikon DS-F2.5 camera or with a WILD M420 macroscope from Leica Microsystems, equipped with a Nikon DS-F2 camera. The imaging software NIS Elements Version 5.02 was used to create multifocus images.

2.2 Morphology and anatomical examination

The training in morphological identification at genus level was given by Dr. Michael Duda (NHMW). It was performed on the basis of the outer shell and essentially followed Glöer's description and illustrations (Glöer, 2002). The following morphological descriptions and drawings (Figure 4) of the shell were taken from Glöer (2002) and translated into English.

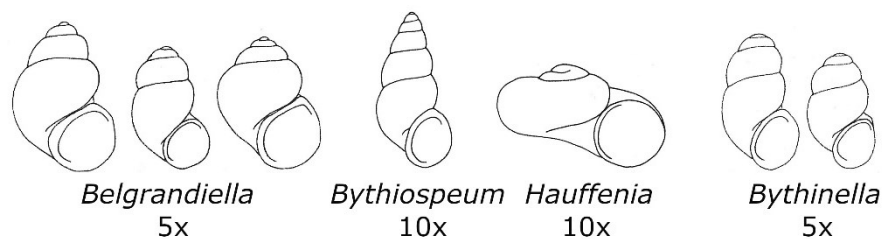


Figure 4: Shells of the genera *Belgrandiella*, *Bythinella*, *Bythiospeum* and *Hauffenia*; modified from Glöer (2002)

The shell of the genus *Belgrandiella* is described as slender cylindrical to ovoid. The margin of the peristome is thickened in the mouth angle and in, or even up to the umbilicus. The margin forms a flat fold with the shell at least in the umbilical region. The operculum is orange to reddish brown.

The shell of the genus *Bythinella* is up to 4 mm high. The shape varies in cylindrical, ovoid and conical forms.

The shells of the genus *Bythiospeum* are yellowish horn-coloured to white, usually finely striped. The peristome margin is continuous, sharp, and sometimes expanded. The spindle margin is turned over. The operculum is deep in the shell.

The genus *Hauffenia* has a valvatoid shell and the protoconch is sculptured with fine wrinkles. The horny, orange-red operculum is almost round with a blunt angle.

For morphological investigations that go beyond the determination of the genus by shell morphology, selected hydrobioid specimens were sent to PD Dr. Martin Haase (University of Greifswald). He was the first to describe the species *B. aulaei* (in the surroundings of the Kalkalpen NP) (Haase et al., 2000) and *Hauffenia wienerwaldensis* Haase, 1992 (Haase, 1992b) and worked out in detail the anatomy of *H. kerschneri* in 1992 (Haase, 1992a). Five putative *B. aulaei* from the OCHS spring and three from the BRUN spring, as well as five supposed *H. wienerwaldensis* from the KREMS spring and five putative *H. kerschneri* from the JÖA spring were sent to him. He was able to confirm these preliminary determinations based on their geographic origin and/or DNA barcode assignment.

To clarify the observed morphological variation of *B. conica*, 20 individuals of the species were dissected by Dr. Michael Duda (NHMW). For this purpose, the animals were first photographed and then placed in 0.5 molar EDTA with a pH of 7.5 for 48 hours. The shell was dissolved while the soft body was preserved at the same time. Then the shell-less soft bodies were converted into 80% ethanol (Verhaegen et al., 2018). The visible structures were photographed. The soft body was dissected with minutiae needles fixed on dissecting needles in order to uncover the genital tract. Illustrations from Boeters and Knebelsberger, 2012 were used for orientation.

2.3 DNA extraction and COI amplification

The investigated taxa are very small organisms, therefore, to obtain sufficient material for the subsequent investigation, the entire organisms were used in the reaction and thus depleted during the DNA extraction. Usually, DNA barcodes from three individuals per spring were generated. To keep one reference individual per sample in the collection, DNA extraction was performed from only three of four individualized and photographed animals. In the case of fewer individuals per sample, one animal was always kept aside (unless there was only one individual), and DNA was extracted from the remaining.

Furthermore, 41 of the 81 hydrobioid specimens of the ABOL Mollusca project were analysed in this study with the same procedure as the specimens from the Kalkalpen NP (see Supporting Figure 1 and Supporting Table 5).

DNA extraction was performed using Qiagen's DNeasy Blood & Tissue Kit following the associated protocol. Lysis was usually carried out for 2.5 hours, in a few cases overnight. Elution was performed twice, each time with 40 µl of elution buffer. DNA concentration was determined by measurement with the Invitrogen Qubit Fluorometer from Thermo Fisher Scientific. The Qubit™ dsDNA HS Assay Kit with the associated standard protocol was used. A list of all individual samples with their respective DNA concentration can be found in Supporting Table 4.

Several attempts were made to optimize the PCR working flow. The Q5® High-Fidelity DNA Polymerase of New England Biolabs and the TopTaq DNA Polymerase of QIAGEN, as well as the QIAGEN Multiplex PCR Kit were tested, the latter one gave the best results and was used further.

For first implementations the primer pairs LCO1490_ABOL_Moll_1/HCO2198_ABOL_Moll_1 (Duda et al., 2017/unpublished) and LCO1490_ABOL_Moll_1/HCO2216_ABOL_Moll_3_pro (Duda et al., 2017/unpublished) of the ABOL Mollusca project were used. However, consistently the best results could be achieved with the primer pair LCO1490_Hydrob1/HCO2216_Hydrob3 (5'-TCAACAAATCATAAGGAYATTGG-3'/5'-CCGGGGAGAATTTAAATATA-3') specially redesigned for this study and optimized for the investigated taxa. For this optimisation process, the alignment of already existing hydrobioid sequences (from the ABOL Mollusca project) was compared with the already existing primers used for sequencing. In the case of wobbles in the primer, it was checked which base was actually selected and this base was used in the new primer to ensure better binding. With the primer pairs LCO1490_ABOL_Moll_1/HCO2216_ABOL_Moll_3_pro and LCO1490_Hydrob1/HCO2216_Hydrob3 a 24 base pair longer piece of the standard DNA barcoding region of COI was amplified. A full protocol of which PCR and sequencing primer were used for which sample can be found in Supporting Table 4.

The QIAGEN Multiplex PCR Kit and the associated manufacturer's protocol was followed. In most cases 1 µl of DNA was used for the PCR, in a few cases where the PCR failed and had to be repeated, 3 µl were used. PCR amplification was performed under the following conditions: 95 °C for 15 min, 35 cycles of (94 °C for 30 s, 48/50 °C (primer pair dependent) for 90 s, and 72 °C for 90 s) and 72 °C for 10 min.

The PCR products were checked on a 1% agarose gel and cleaned with the QIAquick PCR Purification Kit (Qiagen).

PCR products were bidirectionally sequenced by Microsynth Austria GmbH using the PCR primer pairs.

2.4 Data analyses

192 Sequences (Kalkalpen NP and ABOL Mollusca) were assembled, edited and aligned using Geneious Version 10.2.6 (<http://www.geneious.com>, Kearse et al., 2012).

All created DNA barcodes and their associated data like photos, scf files and data spreadsheets (including voucher info, taxonomy, specimen details and collecting data) were uploaded to the Barcode of Life Data system (BOLD) (<https://www.boldsystems.org/>, Ratnasingham and Hebert, 2007). None of the Sequences were flagged, which indicate, that there are no problematic records. All sequences are “Barcode Compliant”. “The standards include a minimum sequence length of 500bp, less than 1% ambiguous bases, the presence of two trace files, a minimum of low trace quality status, and the presence of a country specification in the record as set out by the Consortium for DNA Barcoding (CBOL)” (Milton et al., 2011).

BOLD was used to check which genera and species already have public DNA barcodes, which were then used for comparison with the DNA barcodes generated in this study (status March 2021). In BOLD the sequences are assigned to so-called BINs (Barcode Index Number) (Ratnasingham and Hebert, 2013). The BIN system groups sequences into clusters using well-established algorithms (operational taxonomic units are generated). The BINs are clearly indexed, which means that genetically identical or similar taxa that have been analysed in different studies are stored under common identifiers (BINs). Each new cluster is assigned a globally unique identifier that is registered in the Barcode of Life data system. For more detailed information on how a BIN is formed, see Ratnasingham and Hebert, 2013. With the help of the BIN analysis, it was possible to determine which DNA barcodes were grouped together and with which other species they share the BIN. In addition, the distances within a BIN and between neighbouring BINs can be read off. The BOLD numbers and BINs can be found in Supporting Table 6.

For comparison DNA barcodes from additional specimens and species were used, which were generated in the course of the ABOL Mollusca project (<https://www.abol.ac.at/en/project/molluscs/>), which is currently conducted at the NHMW. A detailed list of this material can be found in Supporting Table 5.

Genetic distance estimations were calculated with Mega version 7 (Kumar et al., 2016) using no variance estimation method, p-distances, uniform rates and pairwise deletion as missing data treatment. Transitions and transversions were included as substitutions.

Nucleotide and haplotype diversities were calculated with DnaSP version 5.10 (Librado and Rozas, 2009).

A Minimum Spanning Haplotype Network (Bandelt et al., 1999) with the available sequences of the genus *Belgrandiella* (ABOL project and this study) was created with PopART version 1.7.

A neighbor joining tree with all hydrobioid sequences of the ABOL Mollusca project and with all sequences of this study was built with Geneious version 10.2.6 (<http://www.geneious.com>, Kearse et al., 2012) (Supporting Figure 1). Default Parameters were used: Tamura-Nei model, 10.000 bootstrap replicates.

BOLD has the option of a tree-based identification for each sequence. This option was used for *B. aulaei* and both *Hauffenia* species from the national park and its surroundings. Since it is not possible to compute bootstrap support within BOLD, the proposed sequences for a tree including all three species have been downloaded (March 2021) and a neighbor joining tree was built with Mega version 7 (Kumar et al., 2016) (Supporting Figure 2). The following default parameters were used: P-distance model, 1000 bootstrap replicates, transitions and transversions as included substitutions, uniform rates and pairwise deletion. Of the sequences BOLD suggested, the cluster with the sequences of the genera *Grossuana* Radoman, 1983, *Islamia* Radoman, 1973, *Daphniola* Radoman, 1973, *Ohridohoratia* Hadžišče, 1959 and *Fissuria* Boeters, 1981 was cut out to ensure a better view. All specimen used are listed in Supporting Table 7.

QGIS version 3.6.1 (QGIS.org, 2020) was used to create all figures of maps, that appear in this thesis (with exception of those with copyright by Management of the Kalkalpen National Park). Layers of Natural Earth, downloaded from www.naturalearthdata.com (September 2019), of OpenStreetMap, downloaded from download.geofabrik.de and of Umweltbundesamt GmbH - data.umweltbundesamt.at, downloaded from www.data.gv.at (September 2019), were used.

The editing of the figures was done by GIMP version 2.10.24 (Revision 2) (<https://www.gimp.org/>) and Inkscape version 0.92.4 (<https://www.inkscape.org/>).

3. Results

3.1 Collecting success and photo documentation

During the present study 39 different springs could be examined, in all of which at least one genus of hydrobioids was found. 35 of these localities contained individuals, which were collected alive and thus tissue for molecular analyses was available. The genus *Bythinella* was found in nearly all springs (36 out of 39), one contained only empty shells. *Hauffenia* was found in 16 springs, only empty shells were found in half of them. The genus *Belgrandiella* could be found in three springs and from all tissue material was available. The genus *Bythiospeum* was found in four springs, all collected shells were empty. Supporting Table 2 lists all springs investigated and genera found. In addition, the table shows detailed information on the collecting events. In 15 localities more than one genus was found. The two genera *Bythinella* and *Hauffenia* were found together in eight springs. The combination of *Bythinella*, *Bythiospeum* and *Hauffenia* was found in four springs (JÖA, REUT, SULZ, Welchau1+2). In two springs (BRUN, PREBL) the genera *Belgrandiella* and *Bythinella* does occur together. The genera *Belgrandiella*, *Bythinella* and *Hauffenia* could only be found together in one spring (OCHS).

The number of shells that was found in one sample varies between one and more than one hundred, depending on the spring, collecting method and genus. In general, higher numbers could be achieved with a net or a scoop, than by hand collecting. Substantially fewer shells were found of the smaller genera *Belgrandiella*, *Bythiospeum* and *Hauffenia*, than of the genus *Bythinella*. While for *Bythinella* mainly living specimens were found, for *Hauffenia* many more empty shells than shells containing tissue were found (alive when collected). For the genus *Belgrandiella* generally only few shells could be found, but these usually contained tissue. Of the genus *Bythiospeum*, with exception of the spring REUT, only few specimens were found in a spring, and all collected shells were empty. The number of all collected shells is also shown in Supporting Table 2.

Overall, 343 photos from 164 individuals were taken during this study. The goal was to create a documentation of the specimen, which can be used as a reference, since for the DNA analysis the whole animals were used. The photos were uploaded to BOLD along with the DNA barcodes to make them available to the public in the future. From the genus *Bythinella* 134 specimen from 27 different localities were photographed from two perspectives (dorsal and ventral). 15 individuals of the genus *Hauffenia* from seven different springs were photographed from three perspectives (dorsal, ventral and lateral), to capture all the important features, such as peristome, umbilicus and apex. From the genus *Belgrandiella*, of nine specimens from three different springs, photos were taken from two perspectives (dorsal and ventral). From the genus *Bythiospeum* six individuals from four different localities were photographed from two perspectives (dorsal and ventral). Figures 5 to 9 show example photos of one specimen from each genus.



Figure 5: Dorsal (left) and ventral (right) view of *Bythinella conica*, individual ABOL_510_3 morphotype 1 from the spring KEHLS; scale 0.5 mm



Figure 6: Dorsal, ventral and lateral (left to right) view of *Hauffenia kerschneri* individual ABOL_512_1 from the spring SULZ 2; scale 0.5 mm



Figure 7: Dorsal, ventral and lateral (left to right) view of *Hauffenia wienerwaldensis* individual ABOL_517_1 from the spring KREMS; scale 0.5 mm



Figure 8: Dorsal (left) and ventral (right) view of *Belgrandiella aulaei* individual ABOL_546_1 from the spring BRUN; scale 0.5 mm



Figure 9: Dorsal (left) and ventral (right) view of *Bythiospeum* cf. *nocki* individual ABOL_548 from the spring Welchau 1+2; scale 0.5 mm

3.2 Morphological identification of species

The determination of hydrobioids is often difficult due to their small size and the lack of diagnostic characters. Based on the size, the shape of the shell and the visibility and colour of the operculum, the snails can be determined at the genus level. Still, the identification of the genus in a juvenile hydrobioid is difficult and cannot be done unambiguously. The most common type of assignment to species level

is based on geographic origin, as these snails often inhabit limited territories (Glöer, 2002). In the rare cases of sympatric occurrences of two species of one genus, anatomical characters must be used (Glöer, 2002). In this study, anatomic examinations were generally not targeted and were performed only in a few individual cases. The most important anatomical features are the genitals (especially the penis due to better discoverability, but also bursa copulatrix, genital opening, oviduct and receptaculum seminis) and the radula (Glöer, 2002).

For the two genera *Bythinella* and *Bythiospeum* two different morphotypes were identified. In the case of *Bythiospeum*, large size differences, and sometimes also differences in the number of whorls within the collected individuals were detected. Between the smaller morphotype 1 (with an estimated size of around 1 mm - no exact measurements were taken in this study) and the large morphotype 2 (with an estimated size of around 1.5 mm and larger) also transitional forms occurred. In the spring Welchau1+2 one of the two individuals found (Figure 9) could rather be assigned to the smaller morphotype (the other individual was damaged, hence no size estimated could be made). The respective individuals of the springs JÖA and SULZ could rather be assigned to the larger form. In the spring REUT both morphotypes could be detected. Out of 50 shells found, 35 were assigned to the large morphotype and 15 to the small morphotype. As an example, Figure 10 shows one individual of the spring REUT, which has a size of around 1 mm and 3 whorls. Figure 11 shows one individual of the spring REUT, which has a size of around 1.6 mm and 4 whorls. The outer shell morphology, as well as the location of the springs indicate that the collected specimens belong to the species *B.nocki*, whose locus typicus is also the spring REUT (Haase et al., 2000).



Figure 10: Dorsal (left) and ventral (right) view of one individual of *Bythiospeum* cf. *nocki* morphotype 1 from the spring REUT; scale 0.5 mm



Figure 11: Dorsal (left) and ventral (right) view of one individual of *Bythiospeum cf. nocki* morphotype 2 from the spring REUT; scale 0.5 mm

In the genus *Bythinella* the two morphotypes have a different aperture. One of the morphotypes has a clear detached peristome (morphotype 1) while the peristome of the other one fits the shell (morphotype 2). Figures 5 and 12 show these differences in one representative of each of the two phenotypes from the spring KEHLS, in which these different morphological features were particularly noticeable. To clarify whether the different morphology corresponds to the sex of the specimen Dr. Michael Duda (NHMW) dissected 10 individuals of each morphotype from the spring. No clear differences in the proportion of one sex was found. For the morphotype 1 six males and four females were determined, for the morphotype 2 the determination was more difficult. Three specimens were determined as females, one as male, the rest as juveniles. So, in both morphotypes males and females could be found. Figures 13 and 14 show a male and a female dissected specimen. To evaluate whether the different morphotypes could be different species, DNA barcodes were generated, and no differences were found (see below). The morphology of the specimens points towards the species *B. conica* and *B. austriaca*, which cannot be distinguished by morphological characteristics (Boeters and Knebelsberger, 2012). The location of the national park indicates that the collected individuals belong to *B. conica* (Boeters and Knebelsberger, 2012; Ternus et al., 2019). DNA barcodes were able to verify this assumption (see below).



Figure 12: Dorsal (left) and ventral (right) view of *Bythinella conica* individual ABOL_510_5 morphotype 2 from the spring KEHLS; scale 0.5 mm

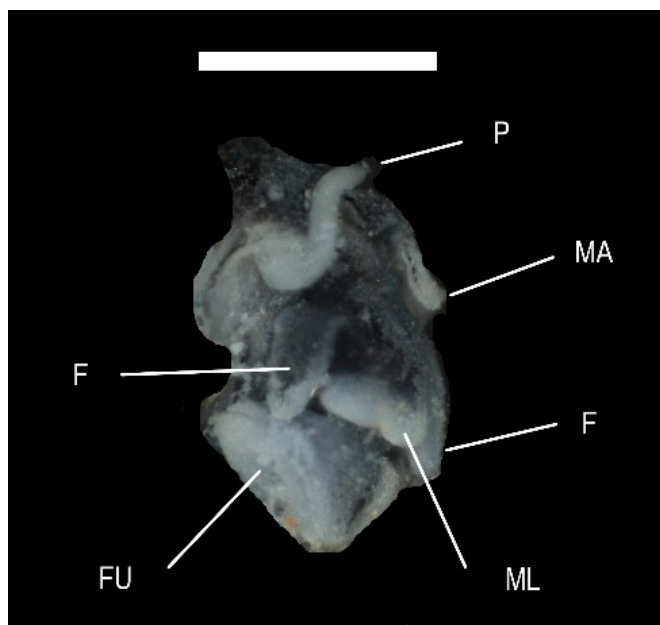


Figure 13: Frontal view of a male of *Bythinella conica* morphotype 1 without mantle from the spring KEHLS; scale 0.5 mm; F: Tentacle, FU: Foot with Operculum, MA: Remains of the mantle, ML: Mouth lobe, P: Penis; photo: Dr. Michael Duda

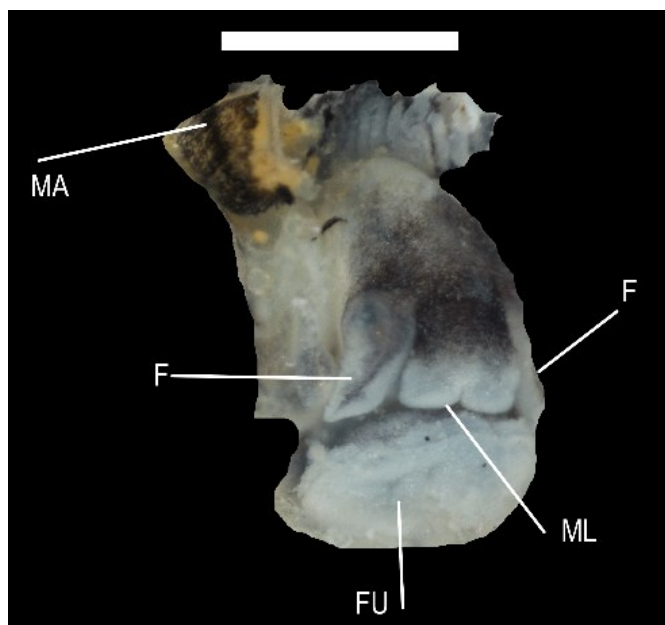


Figure 14: Frontal view of a female of *Bythinella conica* morphotype 1 without mantle from the spring KEHLS; scale 0.5 mm; F: Tentacle, FU: Foot with operculum, MA: Remains of the mantle, ML: Mouth lobe; photo: Michael Duda

Morphological determination of species is especially difficult in the very small genera *Hauffenia* and *Belgrandiella*. There is only few expertise in the determination of Austrian hydrobioids. The collected specimens of the genus *Belgrandiella* resembled the species *B. aulaei*, which was described in the Kalkalpen NP. Further confirmation was achieved by anatomical examinations of eight individuals of two different springs by PD Dr. Martin Haase (University of Greifswald).

At first glance, there were no clear morphological differences in the individuals found of the genus *Hauffenia*. However, the molecular analysis revealed two quite different haplotypes of this genus (see below). Hence, five individuals from the spring KREMS and five from the spring JÖA were determined by PD Dr. Martin Haase (University of Greifswald). He studied the anatomy of the specimen and determined the ones from KREMS as *H. wienerwaldensis* and the ones from JÖA as *H. kerschneri*.

3.3 DNA barcoding success

During this study, DNA was extracted from 111 snails. DNA concentrations ranged from 0.08 ng/μl to 54 ng/μl for the first eluate and from 0.09 ng/μl to 48.6 ng/μl for the second eluate. Some concentrations were too low to measure. From 108 specimens PCR products could be visualized and detected via gel electrophoresis. Three samples of the genus *Bythiospeum*, which were assumed in advance not to contain tissue, unfortunately did not yield positive results. First PCRs were performed with the primer pair LCO1490_ABOL_Moll_1/HCO2216_ABOL_Moll_3_pro of the ABOL Mollusca project. During the study, the primer pair LCO1490_Hydrob1/HCO2216_Hydrob3, which were specially redesigned for this study (for details see chapter Material & Methods) could be implemented and was

then used as the standard procedure. 107 DNA barcodes could be generated (one PCR product failed two times in the sequencing process). According to the quality standards of BOLD, all trace files except from one specimen exhibited a high quality. In the case of the specimen ABOL_532_1, the trace files are of lower quality. Hence, two sequencing operations (with two runs each) were performed and merged, to create an informative sequence. Nevertheless, the ends had to be trimmed more, resulting in a sequence that is about 100 bp shorter than the others. Of the genus *Bythinella* 89 DNA barcodes from 26 different locations, of the genus *Hauffenia* 11 DNA barcodes from seven different locations, and of the genus *Belgrandiella* seven DNA barcodes from three different locations were generated. An overview of all genetically examined individuals with DNA concentrations, BOLD numbers and PCR and sequencing primers can be found in Supporting Table 4.

3.4 Haplotypes of the Kalkalpen National Park

The 89 DNA barcodes of *Bythinella* showed a very low diversity: Mean distance 0.007%, maximum distance 0.29%. Of the 89 DNA barcodes, only two were different at one position (each on a different). The genetic diversity can be calculated with the measures of haplotype diversity (0.05) and nucleotide diversity (0.00007).

The 11 DNA barcodes of the genus *Hauffenia* split into two genetically well differentiated clades. The calculated mean distance within the respective groups is 0.03% in the *H. kerschneri* group (maximum distance is 0.15%) and 0.1% in the *H. wienerwaldensis* group (maximum distance is 0.15%). Within each of the clades one sequence differ in one base. The mean distance between the two groups is 8.08% (minimum distance is 7.88%, maximum distance is 8.51%). Within *H. wienerwaldensis*, the nucleotide diversity is 0.001 and the haplotype diversity is 0.67 (one of the three sequences differs in one position here). The calculated nucleotide diversity as well as the haplotype diversity of *H. kerschneri* is 0, because the one site, where one sequence is different, is excluded during the computing process, as for another sequence there is no information about this site (around 100 bp shorter sequence because of bad quality).

The calculated average distance of the 7 sequences generated from the genus *Belgrandiella* is 0.04% (maximum distance is 0.15%), the individuals are identical except for one (which has one substitution). The haplotype diversity is 0.29 and the nucleotide diversity is 0.0004.

3.5 Sequence comparison with the ABOL Mollusca project

All generated DNA barcodes were compared with the sequences from the hydrobioid species from the ABOL Mollusca project. Since 79 hydrobioid sequences from all over Austria (and two individuals from

Germany) were available in the ABOL project (at the time of the present study), they formed a good comparative database for the species studied here. 41 of these sequences used for comparison were produced during the work of the present study. A full list of the additional material can be found in Supporting Table 5. To get an overview of the clades, a neighbor joining tree with supporting bootstrap values was built from all the sequences (Supporting Figure 1). The tree illustrates the grouping of the genera *Hauffenia*, *Bythinella* and *Belgrandiella* respectively with high bootstrap supports (99 and 100).

The *Bythinella* sequences match those of the species *B. conica*, which were collected from Upper Austria, Lower Austria and Salzburg. Compared to the sequences of the species *B. austriaca*, which has a small genetic distance to *B. conica* and is morphologically indistinguishable (Boeters and Knebelsberger, 2012), most of the sequences are separated by six characteristic substitutions. Overall, the mean distance between the two groups (including all sequences of both projects) is 0.88%, the maximum distance is 0.94% and the minimum distances is 0.59%. Within the *austriaca* group, comprising 24 sequences, the mean distance is 0.02%, the maximum distance is 0.16% and the minimum distance is 0. Within the *conica* group, consisting of 98 sequences (89 of the Kalkalpen NP and its surroundings) the mean distance is 0.01%, the maximum distance is 0.29% and the minimum distance is 0. The tree in Supporting Figure 1 shows, that the clade with the sequences of *B. conica*, *B. austriaca* and *Bythinella cylindrica* (Frauenfeld, 1857) has a supporting value of 100. *B. austriaca* and *B. cylindrica* occur in one clade with a bootstrap value of 92 that is the sister clade to *B. conica*. An overview of all sampling localities of *B. austriaca* and *B. conica* is given in Figure 15. The map shows that the species *B. austriaca* does occur more in the east of Austria, while the species *B. conica* occurs more in the west. This statement agrees with the investigations of Boeters and Knebelsberger, 2012, as well as Ternus et al., 2019.

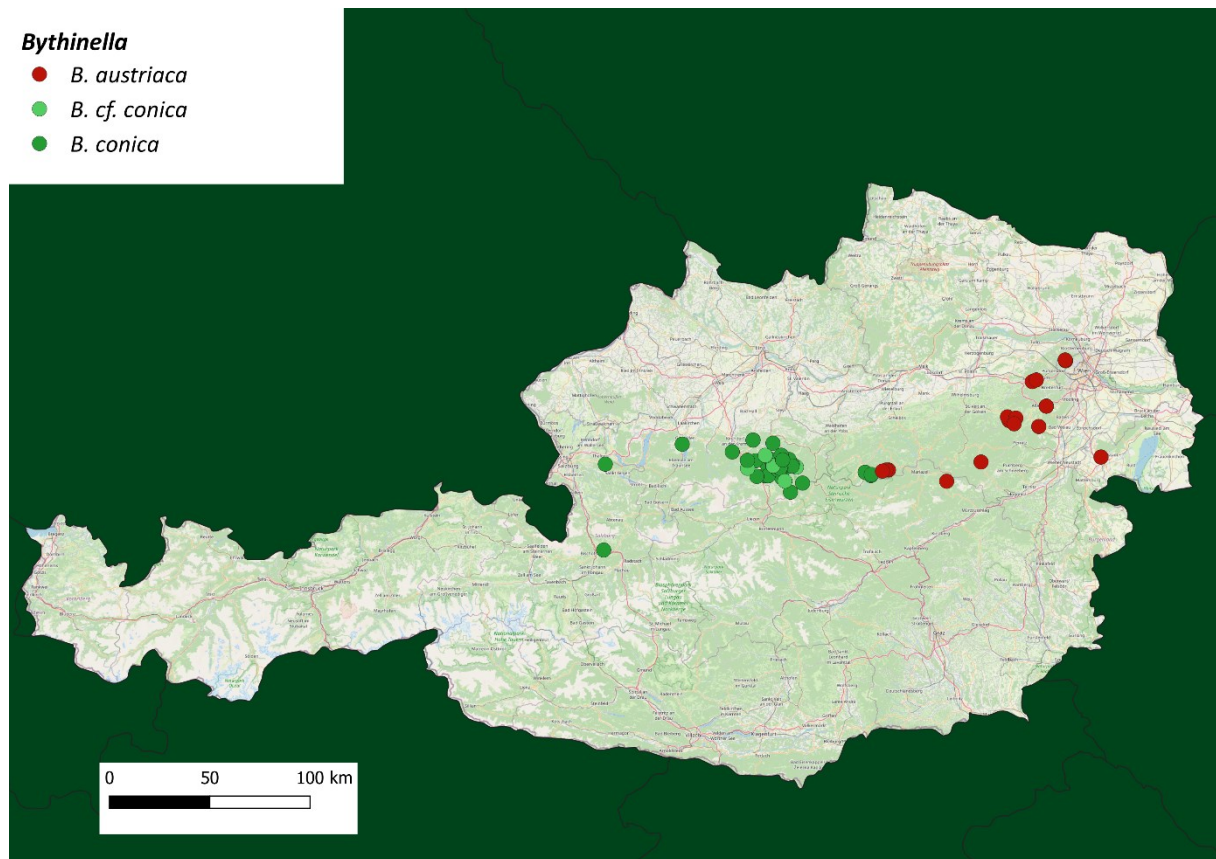


Figure 15: Sample sites of the specimen of the species *Bythinella austriaca* (red dots) and *Bythinella conica* (green dots), which were used for distance analysis. They were collected as a part of the projects ABOL Mollusca and hydrobioids of the Kalkalpen National Park (this study). The light green dots represent sample sites of the Kalkalpen National Park project, from where no DNA barcodes were generated, but based on their sampling locality they are assumed to be the species *Bythinella conica*.

For the sequences of the genus *Hauffenia*, one of the haplogroups from the present study matches perfectly with a sequence of an individual of *H. wienerwaldensis* from Vienna, which was analyzed within the project ABOL Mollusca. Apart from this sequence, no other *Hauffenia* sequences were available for comparison in the ABOL Mollusca project. The two *Hauffenia* species (*H. wienerwaldensis* and *H. kerschneri*) cluster in the tree (Supporting Figure 1) in two clearly separated clades with a bootstrap support of 99.

The sequences of the species *B. aulaei* from the Kalkalpen NP and its surroundings are most similar to some individuals of the species *Belgrandiella fuchsi* (Boeters, 1970) and *Belgrandiella wawrai* Haase, 1996 from Lower Austria, but do not match exactly. In order to make a more precise statement about the comparison of the different haplotypes, a haplotype network of all sequences of *Belgrandiella*, that were generated in both projects was created (Figure 16). The sequences of the individuals from the Kalkalpen NP are separated by four substitutions from the sequences to seven individuals, that were sampled in Kleinzell, Triestingtal, Lilienfeld and Höfnergraben in the Lower Austrian Limestone Alps. Those individuals were determined as *B. fuchsi*, *B. wawrai* and *Belgrandiella sp.*. The sequences

of two individuals from Bad Fischau in Lower Austria, which were determined as *Belgrandiella mimula* Haase, 1996, are separated by 7 substitutions. The greatest distance of 21 substitution is to two individuals from Bad Vöslau, determined as *Belgrandiella parreyssii* (L. Pfeiffer, 1841). The latter also appear in a different BIN on BOLD, which is described below in the next chapter in more detail. In the tree the *B. aulaei* sequences cluster in one clade with a bootstrap value of 94. The map in Figure 17 shows the different sample sites of the *Belgrandiella* sequences, that were available from the ABOL Mollusca project and were used for analysis.

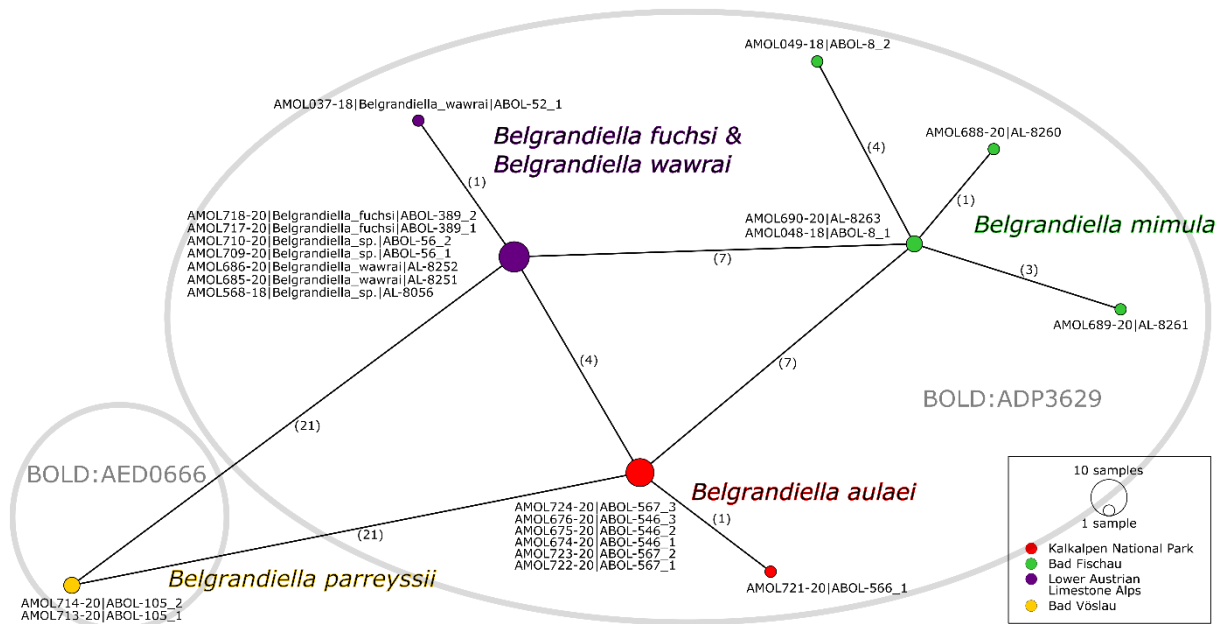


Figure 16: Haplotype Network of all *Belgrandiella* sequences from the present study and from the ABOL Mollusca project. The numbers on the connection lines represent the number of substitutions between the two haplotypes. The different colours indicate different areas, the two grey circles different BINs.

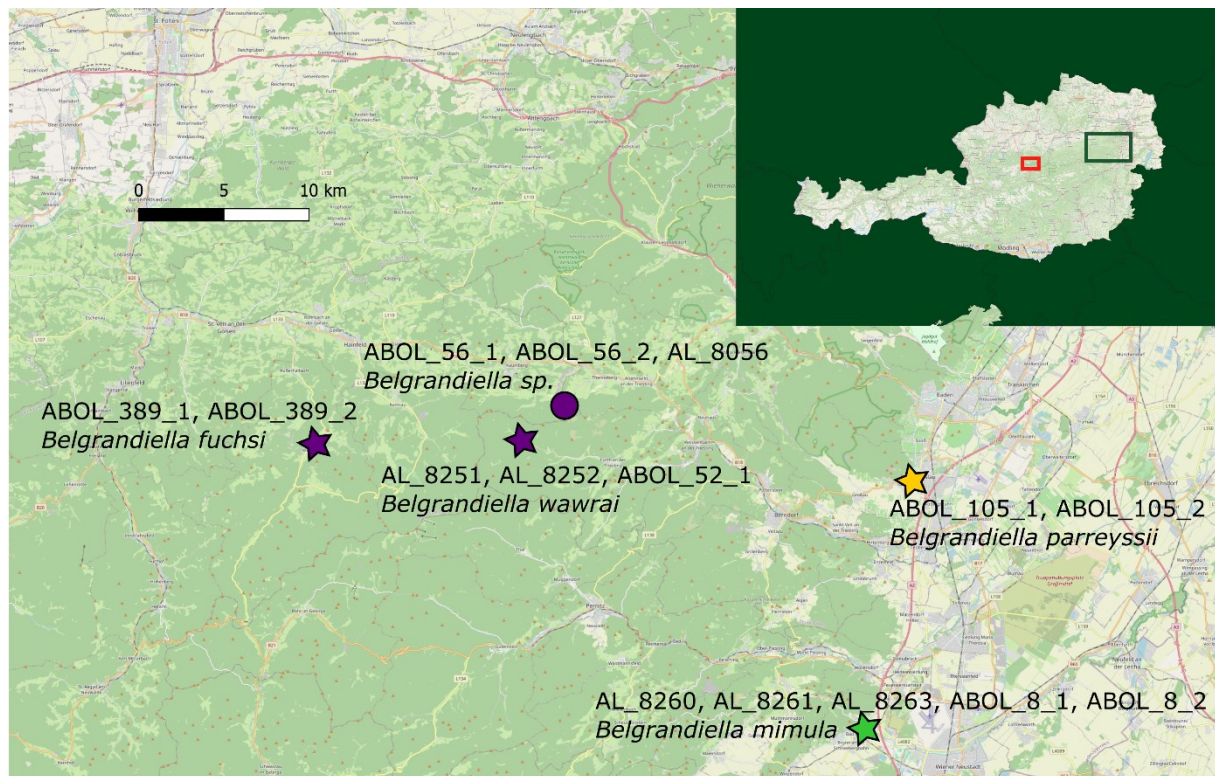


Figure 17: Sample sites of the specimen of the genus *Belgrandiella* from the project ABOL Mollusca, which were used for analysis. The green marks represent the individuals from Bad Fischau, the violet marks represent the individuals from the Lower Austrian limestone alps and the yellow marks represent the individuals from Bad Vöslau. The stars indicate that the location is in the near of the locus typicus. The green square on the Austria map, located in the upper right corner, indicates the approximate area where the collecting sites are located, the red square indicates the position of the Kalkalpen National Park.

3.6 BOLD analysis and Barcode Index Numbers (BINs)

The 107 generated DNA barcodes from the Kalkalpen NP samples were uploaded to BOLD (Barcode of Life Data System) (Ratnasingham and Hebert, 2007), which provides some features for analysis and automatically assigns a BIN (Barcode Index Number) based on sequence similarity to existing sequences in BOLD (Ratnasingham and Hebert, 2013). A list of all uploaded specimens with their corresponding BOLD number and BIN affiliation is to be found in Supporting Table 6.

For all the four investigated genera in this study, COI sequences were available on BOLD. The following numbers were looked up in the middle of June 2021. For the genus *Bythinella* 1394 sequence records from 85 different species are deposited in BOLD, they are assigned to 85 BINs. 122 of them were assigned to the species *B. austriaca* (24 ABOL samples, 98 public), from which 41 specimens were collected in Austria (17 public records, 24 ABOL samples). The remaining specimens come from Poland, Germany, Hungary, Slovakia, Czech Republic. For the species *B. conica*, none other, than the 98 sequences from the ABOL and Kalkalpen NP projects, were available. The genus *Hauffenia* was

represented by 24 COI sequences (from 6 different species), of which 12 came from the ABOL and the Kalkalpen NP project. These were also the only ones collected in Austria and/or belonging to the species *H. wienerwaldensis* or *H. kerschneri*. The 24 sequences are assigned to 10 BINs. For the genus *Belgrandiella* 51 COI sequences from 11 species were available on BOLD and assigned to 3 BINs. 22 of the sequences are from the ABOL and the Kalkalpen NP project and these are the only ones from Austria. 404 COI sequences from 39 species were available for the genus *Bythiospeum* and are assigned to 8 different BINs. 3 of these sequences are from the ABOL Mollusca project. Other 8 (public records) specimens were collected in Austria.

All 89 DNA barcodes of *B. conica* from the Kalkalpen NP were assigned to the BIN BOLD:AAA4467 (see Supporting Table 6). The average distance within this BIN is 0.5%, the maximum 2.61%. The published records of the BIN also contain taxa that have been identified as other species of the genus *Bythinella*: *B. austriaca*, *B. cylindrica* and *Bythinella metarubra* Falniowski, 1987, as well as undefined *Bythinella*. The individuals are from: Poland (37), Germany (18), Austria (17), Slovakia (16), Hungary (15), Czech Republic (4), Unknown (2). The distance to the Nearest Neighbor BIN is 4.4%.

The BIN analysis in BOLD also revealed two different BINs within the generated sequences of genus *Hauffenia*. The average distance within the *H. wienerwaldensis* BIN BOLD:ADP3094 is 0.16%, and the maximum distance is 0.33%. Apart from the sequences from the Kalkalpen NP and the *H. wienerwaldensis* sequence from ABOL, no further sequences are included in the BIN. The average distance within the BIN of *H. kerschneri* BOLD:AEC8473 is 0.03%, the maximum distance is 0.16%. Except for the representatives of the Kalkalpen NP, this BIN does not contain other sequences. No other *H. kerschneri* sequences are deposited in BOLD. The BINs are the Nearest Neighbour BIN of each other with a distance of 8.05%. The BIN, that includes the *H. wienerwaldensis* sequences is also the Nearest Neighbor BIN (BOLD:ADP3094) to two individuals from Slovakia with a distance of 9.03% (BIN BOLD:AAY2140).

The DNA barcodes of *Belgrandiella* from the Kalkalpen NP and its surroundings are assigned to the BIN BOLD:ADP3629. This BIN includes 13 further DNA barcodes from the species *B. mimula* (5), *B. wawrai* (3), *B. fuchsi* (2) and *Belgrandiella sp.* (3), which all came from the ABOL Mollusca project. The average distance within the BIN is 0.9%, and the maximum distance is 2.03%. The distance to the Nearest Neighbor BIN BOLD:AED0666 is 3.29%. The latter BIN consists of the two DNA barcodes from *B. parreyssii* from Bad Vöslau, collected in the course of the ABOL Mollusca project. There are no other sequences of *B. aulaei* in BOLD for comparison.

The neighbor joining tree in Supporting Figure 2 illustrates in a larger frame what the BIN analysis above shows: The two *Hauffenia* clades are separated with a bootstrap value of 75. The next nearest BIN with the *Hauffenia* sequences from Slovakia is separated with a bootstrap value of 97. The *B. aulaei*

cluster together with a bootstrap value of 100 and are within the other *Belgrandiella* group of the Lower Austrian limestone alps. The next nearest BIN with the *B. parreyssii* specimen is separated with a bootstrap value of 100.

3.7 Distribution of Hydrobioids in the Kalkalpen National Park

A full record of the 39 investigated springs of the Kalkalpen NP and all hydrobioids found is listed in Supporting Table 2.

The genus *Bythinella* was found in 36 springs all over the Kalkalpen NP and its surroundings. DNA examinations were performed on individuals from 25 springs, which were consistent with *B. conica*. The map in Figure 18 shows the Kalkalpen NP and its surroundings with all localities where *Bythinella* was found and those from which *B. conica* DNA barcodes were created (stars instead of dots).

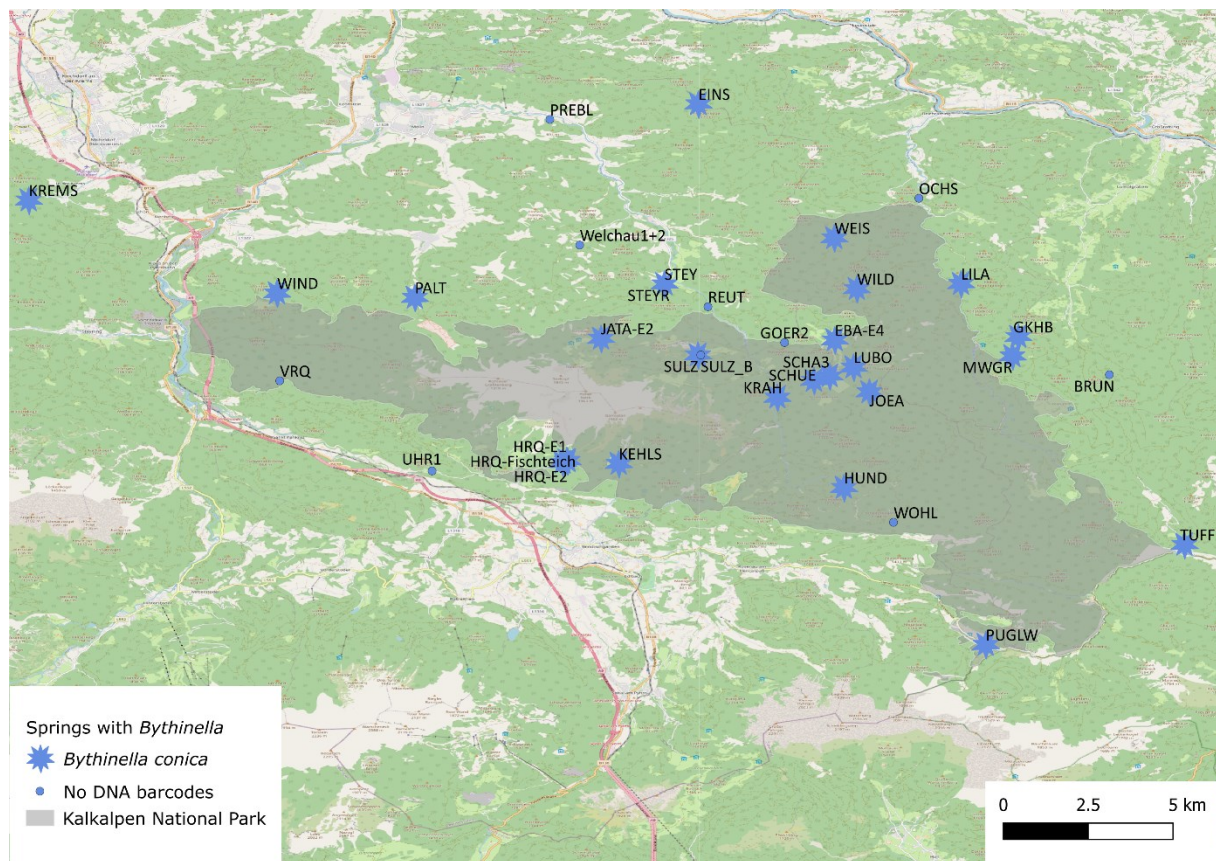


Figure 18: Map of the Kalkalpen National Park and its surroundings. The blue markings indicate the genus *Bythinella* found in the area. Where stars instead of dots are plotted, DNA barcodes were generated and point out the species *Bythinella conica*.

Hauffenia was detected in 16 springs of the national park and its surroundings. In the spring KREMS the species *H. wienerwaldensis* occurs, which can be found in the western surroundings of the park about 5 km outside the border. In six springs (HRQ-E1, HRQ-E2, JÖA, SULZ_B, VRQ, Welchau1+2) the species *H. kerschneri* occurs. Thus, a distribution in the northwest of the park can be established. In the southeastern areas so far, no specimen of the genus *Hauffenia* was found. The distribution of the genus *Hauffenia* in the Kalkalpen NP is depicted in a map (Figure 19). Where DNA barcodes were generated a star, instead of a dot is shown. The white star indicates the species *H. wienerwaldensis*, red stars the species *H. kerschneri*.

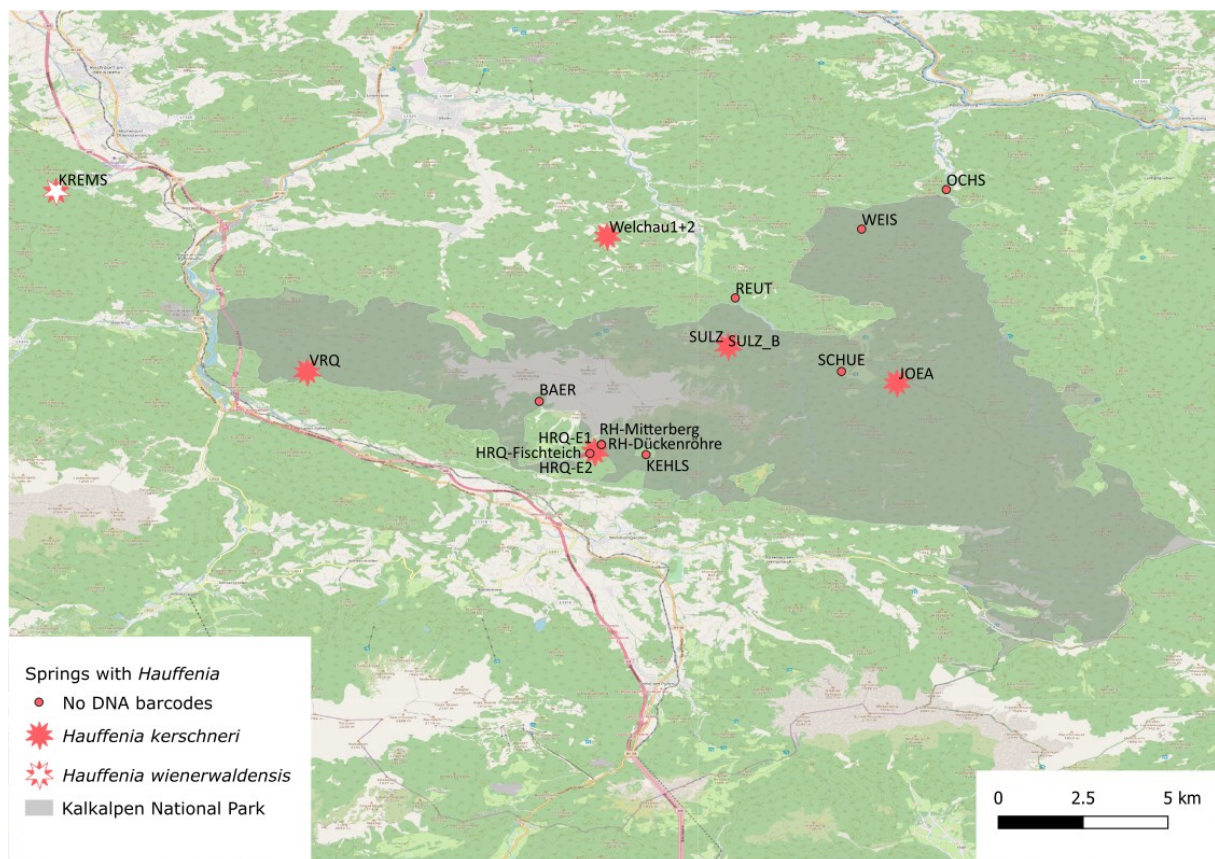


Figure 19: Map of the Kalkalpen National Park and its surroundings. The markings indicate the genus *Hauffenia* found in the area. Where stars instead of dots are plotted, DNA barcodes were generated. The sequences of the localities with red stars are consistent with them of *Hauffenia kerschneri* (determined by PD Dr. Martin Haase, see chapter “Morphological identification of species”). The sequences of the locality with the white star are consistent with them of a *Hauffenia wienerwaldensis* sequence from the ABOL Mollusca project.

B. aulaei was found only outside the Kalkalpen NP. The three springs are located in the north, northeast and east outside the national park and are several kilometres apart from each other. The map in Figure 20 shows the distribution of the species in the surroundings of the Kalkalpen NP.

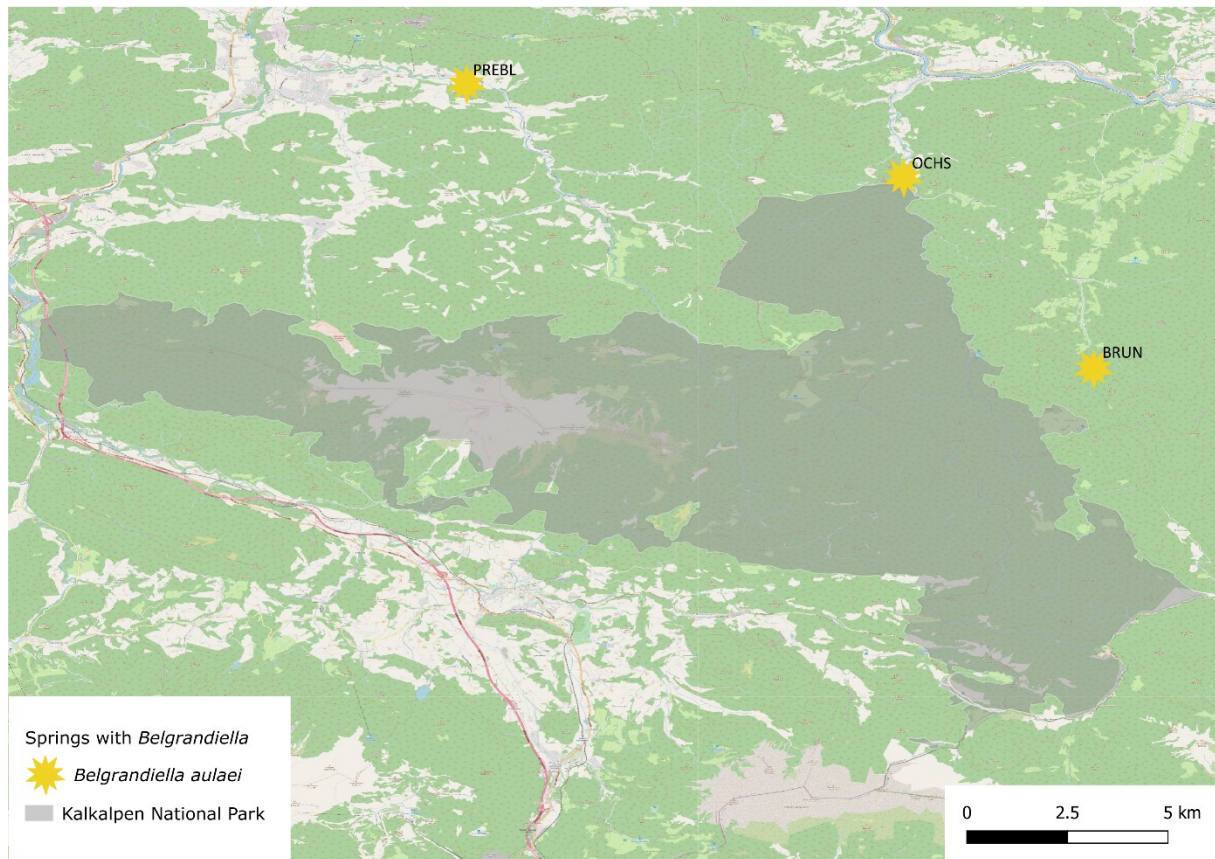


Figure 20: Map of the Kalkalpen National Park and its surroundings. The yellow stars indicate the species *Belgrandiella aulaei* found in the area.

Bythiospeum was found in four springs in the central north of the Kalkalpen NP and its surroundings. Only empty shells of the genus were found. Figure 21 shows a map of all sample sites of *Bythiospeum*.

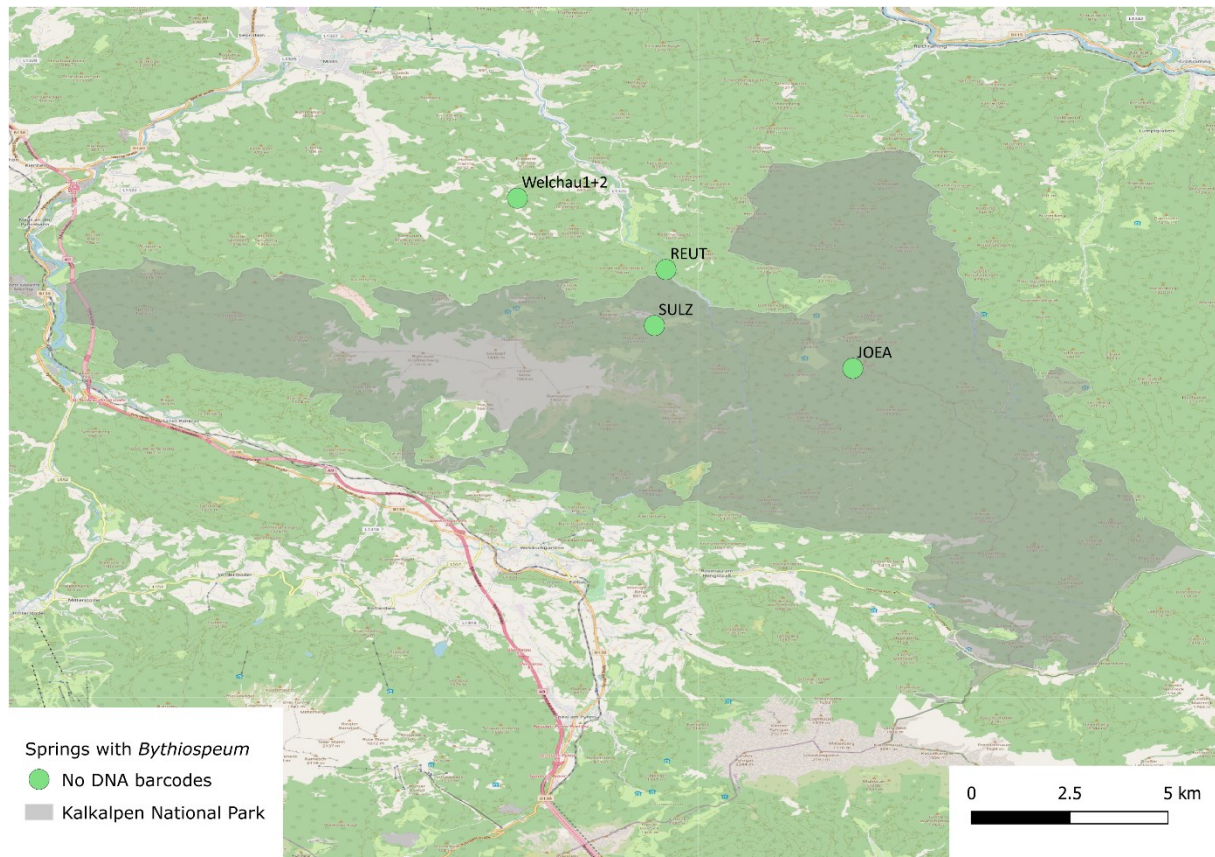


Figure 21: Map of the Kalkalpen National Park and its surroundings. The green dots indicate the genus *Bythiospeum* found in the area. No DNA barcodes could be generated because no live animals could be collected.

4. Discussion

4.1 Discussion of methods

4.1.1 Sampling method

Even though the collecting of the samples was not part of the study conducted at the NHMW in the course of this master's thesis and the various parameters are too diverse to be able to make statistically significant statements, some metadata provided can be assessed at least in part. When it comes to the method of collecting, it is noticeable that a larger number of individuals could rather be collected with scooping or a net than by hand. However, the time that was used for sampling has not been recorded, so it cannot be concluded, which sampling method is mostly efficient. The collecting with sieve and "Triftfalle" is difficult to assess, as collecting with sieve was only used in combination with another method and the "Triftfalle" was only set up at two sites of a spring where no living individuals could be found even with the scooping method. When collecting by hand, a distinction between empty shells and living individuals can be made from the beginning and the number of individuals collected can be defined. The problems here are on one hand the difficulty to find small species and juvenile individuals with the naked eye and secondly to access the springs, because for this method you have to get very close to the site. A combination of two methods seems to be most promising, although it results in more post-processing work. Reischütz et al. (2019, 2020) also recommend the use of a combination of different methods in a study on the mollusc fauna of Furth-Harras, Pottenstein and Tattendorf, when collecting aquatic molluscs mainly by hand and using a net.

At least for the genus *Bythinella*, no significant differences in collecting success were observed in relation to the distance to the spring outlet. The snails could still be found alive even at the greatest distance of about 300 metres. For the other genera, there is too little data available to be able to make a statement. In general, native spring-dwelling snails are better found directly at the outlet than further away. This is especially true for subterranean species, which are only flushed out in exceptional cases (M. Duda, personal communication, July 2021). A possible way to collect subterranean species alive is to hold a fine net or sieve under the outlet of a spring and go in with a brush (preferably extended with a thick wire) and catch everything that comes out (A. Reischütz, personal communication, August 2021). Pumping would also be possible if the surrounding conditions were suitable. Investigating further away still seems useful for monitoring, especially if access is difficult.

4.1.2 Laboratory Work

The well-established workflow of the ABOL Mollusca project could fortunately be adopted in large parts. Living aquatic snails with an operculum tend to retract and seal their shell with the operculum,

which prevents the penetration of alcohol into the tissue and could hinder proper fixation and conservation of the tissue. Consequently, this could cause the degradation of genomic DNA and amplification of the whole DNA barcoding fragment would be difficult. Nevertheless, the DNA extraction, PCR and sequencing of the specimens fixed in 80% EtOH worked very well. One reason for this could be that due to the small size of the snails, the alcohol can still ingress. After optimization of the PCR protocol the low DNA concentration resulting from the small size of the species was not an obstacle in creating DNA barcodes of this group.

DNA barcoding of molluscs might raise difficulties, as their high divergence within COI sequences, may result in mutations in the primer binding region in multiple taxa and hence require adjustments to molecular methods such as primer design (Kruckenhauser et al., 2019). Also, in this study, the primers of the ABOL Mollusca project were adapted for the hydrobioid group. These adapted primers lie somewhat 24 base pairs outside the classical Folmer region and the forward one contains one wobble. The binding of the primers worked well in all analysed taxa, as shown by the high DNA barcoding success, and also facilitates the design of new primer that span through the whole Folmer region.

4.1.3 BOLD analysis

The generated DNA barcodes were all uploaded to the BOLD database. Since none of the sequences were flagged as problematic by BOLD and they all are “Barcode Compliant” (see chapter Material and Methods), the quality of the DNA barcodes can be assumed to be high. However, it should be kept in mind that BOLD does not assess the species identification of the uploaded specimens. In their “The seven deadly sins of DNA barcoding”, Collins and Cruickshank (2012) therefore plead for great care in the a priori determination of species, when adding to reference libraries, such as BOLD. In the current study a great effort was invested to assure correct determination of the specimens, so the uploaded data should serve as good reference DNA barcodes for these species. The automatic mapping of the uploaded DNA barcodes into the BIN system, as well as the generated Taxon ID Tree, provide additional help to discuss species delimitation (discussion of each species is to be found below).

4.2 Species determination and delimitation

The discussion about when a species is a species has been going on for a very long time. There have been many controversies about species concepts and different ways of species delimitation. The most widespread species concepts are the Morphological Species Concept (differentiation by morphological characteristics), the Biological Species Concept (reproducibility with fertile offspring), the Ecological Species Concept (differentiation through ecological niche), and the Phylogenetic Species Concept

(smallest taxon of a phylogenetic reconstruction), but there are many more, e.g., in Zachos, 2016 32 different concepts are listed. The common basic idea of the species concepts is a separately evolving lineage segment, which is seen as the only necessary characteristic in the Evolutionary Species Concept and the Unified Species Concept. These concepts are attempts to define the species category, which Zachos (2016) clearly distinguishes from the species taxon, which is a single species per se. He further distinguishes between species that are given names (taxonomic or T species) and the underlying natural evolutionary species (evolutionary or E species). On delimitation, Zachos writes that species boundaries in nature are "fuzzy" and "making concrete cut-off criteria within a grey area [is] necessarily somewhat arbitrary" (Zachos, 2016). The grey area describes the time of speciation, in which the representatives of a species diverge but no clear new species have yet emerged. The species are in *statu nascendi*. In taxonomy and especially in species delimitation, a distinction is made between splitting and lumping of species. Splitting means dividing a taxon into several (new) taxa. Lumping in contrast, means combining several taxa into one. Often these processes are not based solely on objective criteria but are biased by the taxonomist working on a species group, which is why the terms "lumpers" and "splitters" have become common, describing the respective tendency of taxonomists to rather combine or rather split species. It is important to keep in mind that the decision on whether to split or merge species could have an impact on the conservation of species (Zachos, 2016).

A widely used way of delineating species with DNA barcodes is the use of thresholds. These are calculated by the gap between the largest intraspecific distances and the smallest interspecific distances of a group (=DNA barcoding gap). The existence of such a gap is often questioned in the literature. Also, Collins and Cruickshank (2012) note, that "overlap between intra- and interspecific distances may be the rule, rather than the exception". Moritz and Cicero (2004) list possible restrictions in the use of DNA barcoding in defining species boundaries: "retention of ancestral polymorphism, male-biased gene flow, selection on any mtDNA nucleotide (as the whole genome is one linkage group), introgression following hybridization, and paralogy resulting from transfer of mtDNA gene copies to the nucleus". It is proposed to combine DNA barcoding with other data, such as morphological, anatomical, behavioural, ecological or other molecular data, and to integrate further integrative taxonomic analyses (Kekkonen et al., 2015; Wiemers and Fiedler, 2007). The criticisms listed here for species delimitation by using the COI gene are equally applicable to any other marker, as long as only one is used (the whole mt genome has, due to the lack of recombination, to be considered as one marker). An important point to consider when talking about species differentiation and delimitation using DNA barcoding is made by Moritz and Cicero (2004) in their paper "DNA Barcoding: Promise and Pitfalls": "[...] the very term DNA barcoding is unfortunate, as it implies that each species has a fixed and invariant characteristic—like a barcode on a supermarket product. As evolutionary biologists, we should question this analogy".

Since the hydrobioids are not a group that can be easily distinguished morphologically, due to few and diverse characteristics (see introduction 1.1), some studies have been made to delimit species using genetic data (see also introduction 2.3). While Delicado, 2018 finds that the COI fragment “provides sufficient resolution to detect intra- and interspecific variation in springsnails“, Bichain et al. (2007), who investigated the species delimitation within the genus *Bythinella*, come to the conclusion that the mitochondrial DNA barcoding gene COI alone is not sufficient to identify a species boundary and that the inclusion of other markers is necessary. Wilke et al., who examined the group of hydrobioids phylogenetically in 2013, also states that a “combination of ‘standard’ gastropod genes is very useful for phylogenetic studies targeting family-groups or lower rissooidean taxa“. Haase et al. (2007) advocate especially an integrative approach with morphological, anatomical and genetic studies for species delimitation in the genus *Bythinella*.

For this study, DNA barcoding was mainly used to compare the generated data with existing data (assign unknown specimens to species) and to create (new) genetic references. For the species *B. aulaei* and *H. kerschneri* the specimens, which were used to establish the reference DNA barcodes, were determined morphological and anatomical, which will make it easier to identify these species in the future. For the delimitation of some species, however, the tool of DNA barcoding alone was not sufficient. In order to be able to investigate the differentiation of the species *B. conica* from the species *B. austriaca*, as well as the species *B. aulaei* from other closely related species of the genus, additional investigations with further nuclear markers would be necessary. The same applies to the general investigation of the phylogenetic relationships of the Austrian hydrobioids known so far. In the following, the assessments of the investigated species are discussed.

4.2.1 Assessment of the species *Belgrandiella aulaei*

One of the collecting sites of the species *B. aulaei* is not far from the locus typicus of the species (approx. 7 km). In addition to this, the anatomy of some specimens was examined by the first describer M. Haase himself. These two points and the fact that the generated sequences are almost identical (one different at one position), suggest that the collected snails of this genus represent one species and can be clearly assigned to the *B. aulaei*. An additional analysis of specimens from the locus typicus would complete the picture. For the delimitation of the species to other species of the genus *Belgrandiella* in Austria based on genetic data, the situation has to be discussed in more detail (the morphological delineations can be found at Haase, 1994, 1996; Haase et al., 2000).

In general, it is striking that the intra- and interspecific genetic distances of the Austrian *Belgrandiella* species considered in this study are quite low. The haplotype network in Figure 16 shows this particularly well. Only one substitution in the approx. 670 bp COI fragment of one of seven specimen

shows the intraspecific variance (average distance of 0.04%) of the species *B. aulaei* here. The minimum substitutions to representatives of other species are four (*B. wawrai* and *B. fuchsi*) and seven (*B. mimula*). The largest distance with 21 substitutions is to the species *B. parreyssii*. These short distances are one reason why all these species, except *B. parreyssii* share a BIN in BOLD. The average distance within this BIN, which can be considered as an OTU (operational taxonomic unit), is 0.9%, the maximum distance is 2.03%. The distance to the Nearest Neighbor BIN, which includes exclusively the species *B. parreyssii*, is 3.29%. Various values for the average genetic divergence of the COI gene among species of the family Hydrobiidae can be found in the literature. In their studies on *Pseudamnicola* Paulucci, 1878 species, Delicado et al. (2012) found a mean difference of about 8%. They also list other values of interspecific differences for the family Hydrobiidae from literature: “*Hydrobia* in Wilke, Rolán and Davis 2000, 3-5.5%, and *Floridobia*, 0.5-6.1%, *Marstonia*, 1.0-8.5% and *Pyrgulopsis*, 2.8-11.2% in Hershler et al. 2003”. In 2018, Delicado studied the species of genus *Sadleriana* Clessin, 1890 and found an overall average intraspecific differences of 1.8% in the COI fragment. With Liu et al., who genetically examined the species *Pyrgulopsis kolobensis* (D. W. Taylor, 1987) in 2015, the mean intraspecific divergence was between 0.3% and 2.9%. The inclusion of literature data on the Hydrobiidae family is useful for the discussion of the species status of *B. aulaei*, but it would be unreasonable to derive a general threshold. Falniowski (2018) also recognised in his "Species Distinction and Speciation in Hydrobioid Gastropods" that there is no universal rule and that the level of interspecific distances varies among different genera. The examples mentioned above show, that very small interspecific genetic distances do also occur in other hydrobioids and suggest, that the data on *B. aulaei* do not contradict their species status, however, to clearly verify their species status a larger data set with more samples as well as an investigation with nuclear markers would be necessary.

The clear delimitation of the species *B. parreyssii* is supported, on the one hand because of the separation in the BIN system, on the other hand because of greater genetic distance with similar spatial distance to the other species studied (see Figures 16 and 17). Also, in the NJ trees in the Supporting Figures 1 and 2, this clearly delineated clade is supported with high bootstrap values. Haase (1994) found in his genetic analyses on the basis of allozyme electrophoresis high interspecific distances between *B. fuchsi* and *B. parreyssii*, which is consistent with the data in this study. For the two species *B. wawrai* and *B. fuchsi* no genetic differences were found in the analysis of the present study. In the haplotype network, they occur in the same haplogroup - their generated COI sequences are identical (except for one with one substitution). Anatomically, however, the two species are separated by the location of the bursa copulatrix (Haase, 1996).

One reason for the low genetic distances of the *Belgrandiella* species could be that they are relatively young species that might have formed during the Pleistocene. During the ice ages, many areas of the Eastern Alps were glaciated, but especially in the northeastern and southeastern parts, the ice sheet

was never completely closed and could have served as ice age refugia for these snails. Postglacial recolonisation in Central Europe is also assumed for the hydrobioid genus *Bythiospeum* (Richling et al., 2017). In contrast, Haase (1996) suspects that the Austrian *Belgrandiella* species are rather old, which is not supported by our data. The only exception is *B. parreyssii*, with a genetic distance of approx. 3.29% to the other species, which might have survived the glaciations in a separate refugium. However, given the small sample size and range and the lack of fossil data it appears not justified to carry out a molecular clock analysis.

4.2.2 Assessment of the species *Bythinella conica*

The DNA Barcodes of the different morphotypes of *Bythinella* that were collected in the Kalkalpen NP, were identical and hence give no indication that these morphotypes represent different species. It is known that the intraspecific and interspecific variability of the shell morphology of the genus *Bythinella* can lead to misidentifications (Glöer, 2002). The most likely assumption seems to be that the differences are due to various environmental influences. As early as 1979, the shell variability of the genus was described as ecophenotypic (Falniowski, 1987). Falniowski (2018) also points out that “[...] in springs reproduction takes place throughout a year, but the conditions - like amount of food (e.g. algae) varies between summer and winter, which often results in generations strikingly different in morphology at the same spring, which mimics distinct species.” Another hypothesis was, that the different morphotypes reflects sexual dimorphism, this could be rejected by the anatomical examinations by Dr. Michael Duda (NHMW).

The distribution (Boeters and Knebelsberger, 2012; Ternus et al., 2019), as well as the genetic data of the *Bythinella* specimens clearly assign them to a group of individuals designated as *B. conica*. However, the discussion remains whether this species should be delimited as a separate species, or better regarded as a subspecies of *B. austriaca*, as it is suggested for example by Glöer, 2002. Boeters and Knebelsberger, 2012 even divide the species *B. conica* into two subspecies, one of which is geographically isolated in a small area of the Tiroler Ache and is morphologically distinct. The discussion in the present study refers only to the species level. The most important argument to treat *B. conica* and *B. austriaca* as one species is that they do not differ morphologically and anatomically, so the only differentiation is geographical and genetic (Boeters and Knebelsberger, 2012). An integrative approach as it is suggested by Schlick-Steiner et al. (2010) in general and by Haase et al. (2007) in particular for the genus *Bythinella*, cannot be adhered to. A study on morphometric differences is currently being conducted at the University of Salzburg and may contribute to new insights into external differences of *B. austriaca* and *B. conica* (Ternus et al., 2019).

Already in Boeters and Knebelsberger (2012) a clear genetic distinction in the COI gene between *B. austriaca* and *B. conica* was described. Although the distances were quite low, a distinct gap between the highest intraspecific distances of 0.43% and 0.87% (mean 0.22% and 0.11%) and the lowest interspecific distances of 1.3% (mean 1.5%) was found. The same pattern can be found in the current study, where the mean interspecific distance between the two groups is 0.88%, which is rather low and let the species on BOLD assigned to the same BIN. This value is below the threshold of 1.5%, suggested by Bichain et al. (2007) for the delimitation of *Bythinella* species. Also, the mean intraspecific distances were lower in the present study (*B. conica* 0.01%, *B. austriaca* 0.02%). These differences in the distances between the two works can be explained by the fact that the sequences used by Boeters and Knebelsberger generally have a higher variability.

Boeters and Knebelsberger postulated in 2012 that the species *B. austriaca* is more likely to be distributed in the east of Austria and the species *B. conica* in the west. The data obtained here support this hypothesis (see Figure 15). Like Boeters and Knebelsberger (2012), unfortunately no assumptions can be made about the geographical barrier in the present study, even though the nearest collecting sites of the different haplogroups are only about 4 km apart. The available data does not indicate that isolation by distance is present, as no increasing difference in divergence with increasing geographical distance can be detected.

If *B. conica* and *B. austriaca* are indeed separate species, they could be quite young species that have emerged in the late Pleistocene and until now no major genetic differences have formed. For the genus *Bythinella*, Wilke et al. (2010) found evidence of non-adaptive radiation. In such cases a morphostatic evolution can be recognised, as it is also discussed by Falniowski (2018) for hydrobioids, in which species arise that do not differ from each other either morphologically or ecologically. The question remains whether these are then different species and how distinct the genetic differences would then have to be.

The aim of the current study was not to give a definitive answer whether *B. austriaca* and *B. conica* are to be regarded as separate species. However, data were provided that will be helpful for the discussion about it and it could be shown that DNA barcoding provides a good possibility to distinguish the two taxa.

4.2.3 Assessment of the species *Bythiospeum nocki*

In the course of this study, shells of the genus *Bythiospeum* were (re)found at the locus typicus of the species *B. nocki* (spring REUT), which was described there in 2000 by Haase et al. on the basis of shell morphology. This allows the conclusion that at least the snails collected there belong to the species *B.*

nocki. Based on the distribution the species is also assumed for the remaining findings. One of the localities is also listed as additional material in the first description (spring Welchau) and the other springs SULZ and JOEA are within the radius of about 5 km around the locus typicus, as the additional localities in the first description. Future anatomical and genetic study of living material (which unfortunately was not available in this study) is inevitable to verify this assumption. Even though the spring JOEA belongs to a different catchment area than the other springs, past experience has shown that assuming a new species of the genus *Bythiospeum* due to a different distribution alone can lead to an overestimation of the number of species (Richling et al., 2017).

The assessment of the different morphotypes found is difficult in this case, due to the lack of anatomical and genetic data. It cannot be excluded that they are different species, sex differences or differences in development. Differences in generations, as noted in the genus *Bythinella* (see above), could also be a reason for the different morphotypes.

4.2.4 Assessment of the species *Hauffenia kerschneri* and *Hauffenia wienerwaldensis*

For the genus *Hauffenia* two genetically well differentiated clades in the Kalkalpen NP and its surroundings can be presented as an unexpected result in this study. Until now, no distribution of any species other than *H. kerschneri* was listed in the literature for this region and also no morphological differences were identifiable in the first inspection of the specimens. The mean distance between the two clades is 8.08% (mean intraspecific distances are 0.03% and 0.1%) and thus also lies in the spectrum of the interspecific distances calculated by Rysiewska et al. (2017) for COI in the genus *Hauffenia*. In BOLD, too, the two clades are assigned to different BINs. The respective clusters in the NJ trees in the Supporting Figures 1 and 2 show bootstrap support values of 100%.

PD Dr. Martin Haase (University of Greifswald), who is an expert on Austrian hydrobioids, anatomically examined the specimens sent to him (five each) and identified them as *H. wienerwaldensis* and *H. kerschneri*. For the DNA barcodes generated for the *H. wienerwaldensis* group, there was also a match to one *H. wienerwaldensis* sequence available through ABOL. Which of the two subspecies of *H. kerschneri* presented by Haase in 1992 occurs in the Kalkalpen NP cannot be answered in the present study, this would require a detailed anatomical examination.

The two representatives of the Nearest Neighbor BIN of the two *Hauffenia* groups come from Slovakia (status March 2021) (see also the tree in Supporting Figure 2). However, due to the limited sampling area and number of specimens, for which DNA barcodes of *Hauffenia* are available, it cannot be clearly stated whether this is the closest related taxon.

The reference DNA barcode created for the species *H. kerschneri*, as well as the additional genetic data for the species *H. wienerwaldensis*, of which only one COI sequence was available so far, will be helpful in the future to assign specimens of *Hauffenia* to a species without difficult anatomical examinations.

4.3 Species distribution inside the Kalkalpen National Park

It can be concluded that the species *B. conica* occurs widely in the Kalkalpen NP, because specimens of the genus *Bythinella* were barcoded from various springs throughout the area and all the sequences generated refer to this species. The locations where the species was found include different catchment area (areas from which the water is drawn (Stadler, 2017)) (compare Figure 18 and 22). The same applies to the species *H. kerschneri*, which was mainly collected in the northwest of the park (compare Figure 19 and 22). To clarify whether the species also occurs in the southeast further data is required. The presumed species *B.nocki*, which was found in the central north of the national park, also occurs in at least two different catchment areas (just two sample sites within the park) (compare Figure 21 and 22). Despite the high collecting effort for the species *B. aulaei*, only sites outside the Kalkalpen NP could be identified. Further studies are necessary to say whether a distribution within the park is probable. The same applies to the species *H. wienerwaldensis*, which was only identified in one spring outside the park. If in the future living individuals of the genus *Hauffenia* are found in springs where only empty shells have been found so far, the DNA barcodes can be used to determine which of the two species occurs there.

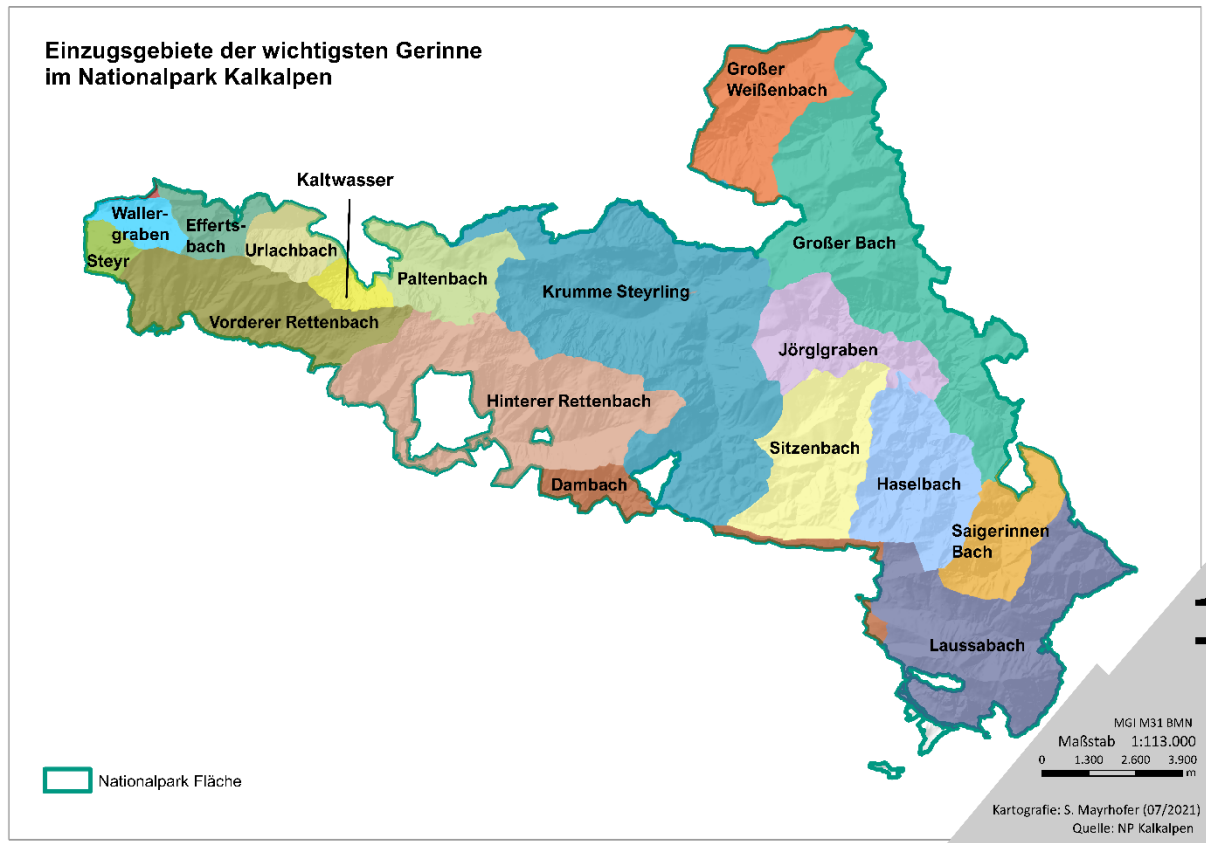


Figure 22: Catchment areas of the Kalkalpen National Park; copyright by Management of the Kalkalpen National Park

Apart from the fact that hydrobioids are habitat specialists (Miller et al., 2018), that require uncontaminated water with relatively low temperatures (Moog, 2002; Wilke et al., 2010), little is known about the ecology of the group (Falniowski, 2018). Wilke et al., (2010) wrote about the genus *Bythinella* that they occur most frequently in rheocene springs. Also, in this study, many individuals of all species were found in rheocene springs (see Supporting Table 1). Even though this is mainly due to the fact that the majority of the collected springs are rheocene, it can be confirmed that many hydrobioids occur in rheocene springs. An exact comparison of the individual parameters (amount of water, temperature, pH value, oxygen level, ions concentrations, turbidity, microbes, etc.) from the monitoring studies of the Kalkalpen NP with the found spring snails exceeds the capacity of this study but should be considered in further studies.

For the species *B. aulaei*, *B. conica*, *B.nocki* and *H. kerschneri*, new localities within the known overall distribution area (Boeters and Knebelsberger, 2012; Haase, 1992a; Haase et al., 2000; Reischütz, 2010a) can be confirmed. For species *H. wienerwaldensis*, the known distribution range (Reischütz, 2010b) could be extended with this study.

4.4 Endangerment and Conservation

All the species investigated in this study are endemics or subendemics (in the case of *B. conica*) (Reischütz and Reischütz, 2007) and require special protection. Their restricted habitat and low ability to disperse (Miller et al., 2018) puts the spring-dwelling snails at additional risk, as contamination of the spring could wipe out the entire population (Strong et al., 2008; Zulka, 2014). Karst systems in particular (around three quarters of the Kalkalpen NP are karstified) are often in direct contact with the surface, so that small amounts of soil contamination are carried to the spring by aquifers (Stadler, 2017). Strong et al. (2008) lists a variety of potential risks of hydrobioids, including “[...]depletion of ground water for a number of urban and rural uses including water capture for stock, irrigation or mining, spring or landscape modification and trampling by cattle[...]” as well as “[...]gravel mining and other sources of mine waste pollution, dredging, channelization, siltation from agriculture and logging, pesticide and heavy metal loading, organic pollution, acidification, salination, waterborne disease control, urban and agricultural development, unsustainable water extraction for irrigation, stock and urban use, competition and/or smothering from introduced species [...]”.

Although the special endangerment of the hydrobioids within the Kalkalpen NP was already recognised before (Jaksch and Steger, 2014), this study has contributed significantly to the knowledge of these endemics in the park. This helps to determine the localities that need to be specially protected and can act as model study for future monitoring projects. It also provides an opportunity to review existing protection measures and discuss updates. By prioritising its conservation goods, the Kalkalpen NP determines which species and habitat types require special protection (Nationalpark O.ö. Kalkalpen Ges.m.b.H, 2018). The protection of springs from grazing cattle is fulfilled, for example, through fences (annual assembly and disassembly necessary) and watering places for the cattle (Nationalpark O.ö. Kalkalpen Ges.m.b.H, 2018; Weigand, 2008). In the future, regular, long-term monitoring should be considered in order to evaluate the changes in the various springs in the coming years. More in-depth studies, especially on the genera *Hauffenia*, *Belgrandiella* and *Bythiospeum*, would be highly recommended in any case. In addition, an extension of the area of the Kalkalpen NP to the sensitive marginal areas worthy of protection should be proposed to protect the species *B. aulaei*, which is so far only known from the surroundings of the national park. If no area extensions are possible, other protection of individual springs outside the national park should be implemented (e.g. as natural monuments).

5. Conclusio

The occurrence of hydrobioids in the Kalkalpen NP were recorded in the present study and new genetic data could be generated for some representatives of this group. The presence of five different hydrobioid species can be confirmed: *B. aulaei*, *B. conica*, *B.nocki*, *H. kerschneri* and *H. wienerwaldensis*. *B. aulaei* is only rarely found (in three springs) and occurs only outside the boundaries of the national park. For this species, new genetic data were acquired, and reference DNA barcodes were created. The species *B. conica* is present in the entire park and shows a variability in shell morphology but nearly no intraspecific genetic variability. The species *B.nocki* was found only in four springs. It also shows morphological variability in size and shape; genetic data is not available. The occurrence of two different species of the genus *Hauffenia* in the Kalkalpen NP and its surroundings was proven: *H. kerschneri* and *H. wienerwaldensis*. The genetic differentiation between the two species is quite high. For *H. kerschneri*, it was also possible to establish reference DNA barcodes.

The data obtained answers questions from previous studies, regarding which hydrobioids species occur in the national park (Steger, 2012; Weigand, 2012). Data on the first description of *B. aulaei* and *B.nocki* (Haase et al., 2000) could be completed: Further localities were identified and genetic data for the species *B. aulaei* could be obtained. The genetic and geographical differences found between *B. conica* and *B. austriaca* by Boeters and Kneibelsberger (2012) was confirmed by the current data. The previously known distribution range of the species *H. wienerwaldensis* (Reischütz, 2010b) can be reassessed according to the findings of this study. This present study provides genetic data to some of the species of the genus *Belgrandiella* in Austria studied by Haase 1996. All these data contribute to the protection of the endangered group of hydrobioids, which includes many endemic species (Reischütz and Reischütz, 2007) and can quickly become extinct without special protection (Zulka, 2014).

The present study can be used as a model study for further monitoring on hydrobioids. The newly acquired knowledge can facilitate future assignment of hydrobioids in the Kalkalpen NP and in other parts of the distribution range of the studied species.

Although the study has provided an overview of the national park's hydrobioid species, there are still questions that need to be answered by further research. For further knowledge about the species *B.nocki*, it would be important to find living individuals. The same applies to individuals of the genus *Hauffenia* from the springs from which only empty shells could be recovered. Without living material, it is not possible to clearly determine which of the two *Hauffenia* species occurs here. For this approach, the use of eDNA analysis could be tested to obtain DNA from individuals that are not found in the spring but live subterranean. An evaluation of the water parameters related to the hydrobioid occurrences in the monitoring springs of the Kalkalpen NP would also be interesting to increase the

knowledge about the ecology of this group, about which very little is known so far (Falniowski, 2018). The analysis of several nuclear genes would be necessary for the delimitation of the putative species *B. conica* from its sister *B. austriaca* on the one hand and to re-evaluate the delimitations of all Austrian *Belgrandiella* species on the other hand. Furthermore, the phylogenetic study of Austrian hydrobioid species and their status in the European context would need further investigations.

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Supporting Information

Supporting Table 1: Hydrobioids of Austria. The list was taken from Reischütz and Reischütz, 2007 and shortened. CR= Critically Endangered, DD= Data Deficient, EN= Endangered, EX= Extinct, NE= Not Evaluated, NT= Near Threatened, VU= Vulnerable

Austrian Hydrobioids (abridged from Reischütz and Reischütz, 2007)			
Species	IUCN Red List Categories	Endemic	Subendemic
<i>Alzoniella hartwigschuetzi</i>	NT	x	
<i>Belgrandiella aulaei</i>	CR	x	
<i>Belgrandiella austriana</i>	CR	x	
<i>Belgrandiella boetersi</i>	EX	x	
<i>Belgrandiella fuchsi</i>	CR	x	
<i>Belgrandiella ganslmayri</i>	CR	x	
<i>Belgrandiella kreisslorum</i>	EX	x	
<i>Belgrandiella mimula</i>	CR	x	
<i>Belgrandiella multiformis</i>	CR	x	
<i>Belgrandiella parreyssii</i>	CR	x	
<i>Belgrandiella pelerei</i>	CR	x	
<i>Belgrandiella styriaca</i>	CR	x	
<i>Belgrandiella wawrai</i>	CR	x	
<i>Bythinella austriaca austriaca</i>	NT		
<i>Bythinella austriaca conica</i>	CR		x
<i>Bythinella bavarica</i>	CR		
<i>Bythinella cylindrica</i>	CR	x	
<i>Bythinella opaca</i>	NT		x
<i>Bythiospeum wiaaiglica</i>	EX	x	
<i>Bythiospeum bormanni</i>	CR	x	
<i>Bythiospeum cisterciensorum</i>	CR	x	
<i>Bythiospeum elseri</i>	CR	x	
<i>Bythiospeum excelsior</i>	DD	x	
<i>Bythiospeum excessum</i>	DD	x	
<i>Bythiospeum geyeri</i>	EN	x	
<i>Bythiospeumnocki</i>	CR	x	
<i>Bythiospeum noricum</i>	CR	x	
<i>Bythiospeum pfeifferi</i>	CR	x	
<i>Bythiospeum reispense</i>	CR	x	
<i>Bythiospeum tschapecki</i>	EX	x	
<i>Graziana adlitzensis</i>	CR	x	
<i>Graziana klagenfurtensis</i>	CR	x	
<i>Graziana lacheineri</i>	NT		
<i>Graziana pupula</i>	EN		x
<i>Hauffenia danubialis</i>	EN	x	
<i>Hauffenia kerschneri loichiana</i>	CR	x	
<i>Hauffenia kerschneri kerschneri</i>	CR	x	
<i>Hauffenia nesemanni</i>	CR	x	

Species	IUCN Red List Categories	Endemic	Subendemic
<i>Hauffenia wienerwaldensis</i>	CR	x	
<i>Iglica gratulabunda</i>	CR	x	
<i>Iglica kleinzellensis</i>	CR	x	
<i>Potamopyrgus antipodarum</i>	NE		
<i>Lithoglyphus naticoides</i>	VU		

Supporting Table 2: Springs of the Kalkalpen National Park including information about containing hydrobioids, locality details and collecting events.

Springs and species found					
Nbr.	Spring	Genera found	Empty shells	Collected alive	DNA barcodes
1	BÄR	<i>Hauffenia</i>	2x <i>Hauffenia</i>		
2	BRUN	<i>Belgrandiella</i> ; <i>Bythinella</i>		10x <i>Belgrandiella</i> ; 24x <i>Bythinella</i>	3x <i>B. aulaei</i>
3	EBA-E4	<i>Bythinella</i>		21x <i>Bythinella</i>	3x <i>B. conica</i>
4	EINS	<i>Bythinella</i>		35x <i>Bythinella</i>	3x <i>B. conica</i>
5	GKHB	<i>Bythinella</i>		16x <i>Bythinella</i>	3x <i>B. conica</i>
6	GÖR2	<i>Bythinella</i>		9x <i>Bythinella</i>	
7	HRQ-E1	<i>Bythinella</i> ; <i>Hauffenia</i>	23x <i>Bythinella</i> ; >100x <i>Hauffenia</i>	23x <i>Bythinella</i> ; 3x <i>Hauffenia</i>	4x <i>B. conica</i> ; 1x <i>H. kerschneri</i>
8	HRQ-E2	<i>Bythinella</i> ; <i>Hauffenia</i>	5x <i>Bythinella</i> ; 4x <i>Hauffenia</i>	11x <i>Bythinella</i> ; 2x <i>Hauffenia</i>	3x <i>B. conica</i> ; 1x <i>H. kerschneri</i>
9	HRQ-Fischteich	<i>Bythinella</i> ; <i>Hauffenia</i>	100x <i>Bythinella</i> ; >100x <i>Hauffenia</i>	1x <i>Bythinella</i>	1x <i>B. conica</i>
10	HUND	<i>Bythinella</i>		43x <i>Bythinella</i>	3x <i>B. conica</i>
11	JATA-E2	<i>Bythinella</i>		20x <i>Bythinella</i>	3x <i>B. conica</i>
12	JÖA	<i>Bythinella</i> ; <i>Bythiospeum</i> ; <i>Hauffenia</i>	>100x <i>Bythinella</i> ; 1x <i>Bythiospeum</i> ; >100x <i>Hauffenia</i>	56x <i>Bythinella</i> ; 80x <i>Hauffenia</i>	3x <i>B. conica</i> ; 3x <i>H. kerschneri</i>
13	KEHLS	<i>Bythinella</i> ; <i>Hauffenia</i>	80x <i>Bythinella</i> ; 25x <i>Hauffenia</i>	40x <i>Bythinella</i> (10x Morphe1, 20x Morphe2)	7x <i>B. conica</i>
14	KRAH	<i>Bythinella</i>		40x <i>Bythinella</i>	3x <i>B. conica</i>
15	KREMS	<i>Bythinella</i> ; <i>Hauffenia</i>	20x <i>Bythinella</i> ; 25x <i>Hauffenia</i>	5x <i>Bythinella</i> ; 14x <i>Hauffenia</i>	3x <i>B. conica</i> ; 3x <i>H. wienerwaldensis</i>
16	LILA	<i>Bythinella</i>		30x <i>Bythinella</i>	3x <i>B. conica</i>
17	LUBO	<i>Bythinella</i>		26x <i>Bythinella</i>	3x <i>B. conica</i>
18	MWGR	<i>Bythinella</i>		19x <i>Bythinella</i>	3x <i>B. conica</i>
19	OCHS	<i>Belgrandiella</i> ; <i>Bythinella</i> ; <i>Hauffenia</i>	4x <i>Belgrandiella</i> ; 13x <i>Bythinella</i> ; 15x <i>Hauffenia</i>	25x <i>Belgrandiella</i> ; 42x <i>Bythinella</i>	3x <i>B. aulaei</i>
20	PALT	<i>Bythinella</i>		17x <i>Bythinella</i>	3x <i>B. conica</i>
21	PREBL	<i>Belgrandiella</i> ; <i>Bythinella</i>		2x <i>Belgrandiella</i> ; 7x <i>Bythinella</i>	1x <i>B. aulaei</i>
22	PUGLW	<i>Bythinella</i>		22x <i>Bythinella</i>	3x <i>B. conica</i>
23	REUT	<i>Bythinella</i> ; <i>Bythiospeum</i> ; <i>Hauffenia</i>	1x <i>Bythinella</i> ; 50x <i>Bythiospeum</i> (ca. 35x Morphe1 (groß), ca. 15x Morphe2 (klein)); >100x <i>Hauffenia</i>		
24	RH-Dückeröhre	<i>Hauffenia</i>	>100x <i>Hauffenia</i>		

Nbr.	Spring	Genera found	Empty shells	Collected alive	DNA barcodes
25	RH-Mitterberg	<i>Hauffenia</i>	7x <i>Hauffenia</i>		
26	SCHA3	<i>Bythinella</i>		50x <i>Bythinella</i>	3x <i>B. conica</i>
27	SCHÜ	<i>Bythinella</i> ; <i>Hauffenia</i>	>100x <i>Bythinella</i> ; >100x <i>Hauffenia</i>	>100x <i>Bythinella</i> ; 1x <i>Hauffenia</i>	7x <i>B. conica</i>
28	STEY	<i>Bythinella</i>	14x <i>Bythinella</i>	>100x <i>Bythinella</i>	
29	STEYR	<i>Bythinella</i>		20x <i>Bythinella</i>	4x <i>B. conica</i>
30	SULZ	<i>Bythinella</i> ; <i>Bythiospeum</i> ; <i>Hauffenia</i>	>100x <i>Bythinella</i> ; 1x <i>Bythiospeum</i> ; >100x <i>Hauffenia</i>	90x <i>Bythinella</i>	3x <i>B. conica</i>
31	SULZ_B	<i>Bythinella</i> ; <i>Hauffenia</i>	50x <i>Bythinella</i> ; 50x <i>Hauffenia</i>	22x <i>Bythinella</i> ; 2x <i>Hauffenia</i>	1x <i>H. kerschneri</i>
32	TUFF	<i>Bythinella</i>	8x <i>Bythinella</i>	18x <i>Bythinella</i>	3x <i>B. conica</i>
33	UHR1	<i>Bythinella</i>		38x <i>Bythinella</i>	
34	VRQ	<i>Bythinella</i> ; <i>Hauffenia</i>	7x <i>Bythinella</i> ; 8x <i>Hauffenia</i>	11x <i>Bythinella</i> ; 2x <i>Hauffenia</i>	1x <i>H. kerschneri</i>
35	WEIS	<i>Bythinella</i>	20x <i>Bythinella</i> ; 100x juvenile <i>Bythinella</i>	>100x <i>Bythinella</i>	3x <i>B. conica</i>
36	Welchau1+2	<i>Bythinella</i> ; <i>Bythiospeum</i> ; <i>Hauffenia</i>	90x <i>Bythinella</i> ; 4x <i>Hauffenia</i> ; 2x <i>Bythiospeum</i>	55x <i>Bythinella</i> ; 2x <i>Hauffenia</i>	1x <i>H. kerschneri</i>
37	WILD	<i>Bythinella</i>	13x <i>Bythinella</i> ; 15x juvenile <i>Bythinella</i>	15x <i>Bythinella</i>	1x <i>B. conica</i>
38	WIND	<i>Bythinella</i>		33x <i>Bythinella</i>	3x <i>B. conica</i>
39	WOHL	<i>Bythinella</i>		20x <i>Bythinella</i>	

Locality								
Nbr.	Spring	Lat	Lon	Height [m asl]	National park area	Locality	Mountains	Drainage direction
1	BÄR	47.8	14.3	1 385	nature zone	Bärenriedlau	Sengsengebirge	unknown
2	BRUN	47.8	14.5	664	outside park (ca. 2km)	Hirschkogelsattel west	Hintergebirge	Brunnbach
3	EBA-E4	47.8	14.4	1 059	conservation area	Ebenforstalm	Hintergebirge	Großer Bach
4	EINS	47.9	14.4	880	outside park	Einsiedlerkogel west, at Forststraße	Mollner Berge	Roszbach-Krumme Steyrling-Steyr
5	GKHB	47.8	14.5	636	slightly outside park	Großer Bach	Hintergebirge	Großer Bach
6	GÖR2	47.8	14.4	1 075	N/A	Göritz	Hintergebirge	Krumme Steyrling-Steyr
7	HRQ-E1	47.8	14.3	640	nature zone	Hinteres Rettenbachtal	Sengsengebirge	Hinterer Rettenbach-Steyr
8	HRQ-E2	47.8	14.3	639	nature zone	Hinteres Rettenbachtal	Sengsengebirge	Hinterer Rettenbach-Steyr
9	HRQ-Fischteich	47.8	14.3	605	nature zone	Hinteres Rettenbachtal	Sengsengebirge	Hinterer Rettenbach-Steyr
10	HUND	47.7	14.4	1 062	nature zone	Stöffelam, Hundseckstraße	Hintergebirge	Großer Bach
11	JATA-E2	47.8	14.3	1 415	conservation area	Feichtaualm	Sengsengebirge	Krumme Steyrling

Nbr.	Spring	Lat	Lon	Height [m asl]	National park area	Locality	Mountains	Drainage direction
13	KEHLS	47.8	14.3	759	private enclave inside park	Hinteres Rettenbachtal	Sengsengebirge	Hinterer Rettenbach- Steyr
14	KRAH	47.8	14.4	678	probably nature zone	near Krahalm, Bodinggraben	Sengsengebirge	Krumme Steyrling- Oberlauf
15	KREMS	47.9	14.1	629	outside park	Kremstal	Kremsmauer	Krems
16	LILA	47.8	14.5	456	slightly outside park	Großer Bach	Hintergebirge	Großer Bach
17	LUBO	47.8	14.4	1299	conservation area	Schaumbergalm	Hintergebirge	Jörglgrabenbach -Hasel-Großer Bach- Reichramingbac h-Enns
18	MWGR	47.8	14.5	597	slightly outside park	Großer Bach	Hintergebirge	Großer Bach
19	OCHS	47.9	14.5	391	N/A	at Großen Bach, at the height of Weißenbach, at the bottom of the Ochsenkogel	Reichraminger Hintergebirge	N/A
20	PALT	47.8	14.3	501	slightly outside park	Hopfing, just before military training area	Sengsengebirge	Paltenbach-Steyr
21	PREBL	47.9	14.3	420	outside park	N/A	outlet of spring at Krummen Steyrling (Annasberg at Molln-Rabach)	N/A
22	PUGLW	47.7	14.5	836	N/A	Puglalm	Sengsengebirge	Rotkreuzbach- Laussabach-Enns
23	REUT	47.8	14.4	594	nature zone	N/A	Sengsengebirge	N/A
24	RH- Dückeröhre	47.8	14.3	671	nature zone	Rettenbachhöhle in the rear part of the Rettenbachtal at Windischgarsten	Sengsengebirge	Fischbach- Hinterer Rettenbach- Steyr
25	RH- Mitterberg	47.8	14.3	671	nature zone	Rettenbachhöhle in the rear part of the Rettenbachtal at Windischgarsten	Sengsengebirge	Fischbach- Hinterer Rettenbach- Steyr
26	SCHA3	47.8	14.4	1 206	conservation area	Schaumbergalm	Hintergebirge	Krumme Steyrling-Steyr
27	SCHÜ	47.8	14.4	1 116	conservation area	Schaumbergalm	Hintergebirge	Krumme Steyrling-Steyr
28	STEY	47.8	14.4	760	many kilometers outside park	N/A	Totes Gebirge	N/A
29	STEYR	47.8	14.4	537	outside park, catchment area inside park	Wohlführeralm	Hintergebirge	Krumme Steyrling-Steyr
30	SULZ	47.8	14.4	973	conservation area	Zagglbaueralm	Sengsengebirge	Krumme Steyrling-Steyr
31	SULZ_B	47.8	14.4	961	probably conservation area	Zagglbaueralm	Sengsengebirge	Krumme Steyrling-Steyr
32	TUFF	47.7	14.6	576	slightly outside park	Laussabauer	Hintergebirge	Mooshöhebach- Laussabach-Enns

Nbr.	Spring	Lat	Lon	Height [m asl]	National park area	Locality	Mountains	Drainage direction
33	UHR1	47.8	14.3	N/A	slightly outside park	Bründl at the street (Rettenbachtal), south slope Gsperrberg (865m)	Gsperrberg	Hinterer Rettenbach-Steyr
34	VRQ	47.8	14.2	453	nature zone, park boundary	N/A	Sengsengebirge	N/A
35	WEIS	47.8	14.4	473	N/A	Großweißenbachtal	Hintergebirge	Großer Weißenbach-Reichramingbach-Enns
36	Welchau1+2	47.8	14.3	529-584	slightly outside park	Welchau/Welchau-Seitental	Mollner Berge	Krumme Steyrling-Steyr
37	WILD	47.8	14.4	801	probably nature zone	Wilder Graben	Hintergebirge	Wilder Grabenbach-Großer Bach-Reichramingbach-Enns
38	WIND	47.8	14.2	839	probably inside park	Almweide east Windberg at Spering	Sengsengebirge	Effertsbach-Steyr
39	WOHL	47.7	14.4	N/A	nature zone	Wohlführeralm	Hintergebirge	Sitzenbach-Gr.Bach

Additional information about springs and collecting events

Nbr.	Spring	Monitoring spring	Type of spring	Distance from the spring outlet [m]	Nbr. of collectings	Collecting dates	Collecting method	Collector
1	BÄR	yes	rheocene - moss helocene	1-3	1	15.11.2018	scooping	Weigand E.
2	BRUN	no	rheocene swamp spring	0-5	1	17.10.2018	by hand	Weigand E.
3	EBA-E4	yes	spring brook	ca. 300	1	08.10.2018	by hand	Weigand E.
4	EINS	no	probably rheocene and moss-helocene	N/A	1	26.10.2018	by hand	Weigand E.
5	GKHB	yes	rheocene	ca. 50	1	15.10.2018	by hand	Weigand E.
6	GÖR2	yes	rheocene	3-15	1	21.11.2018	by hand	Weigand E.
7	HRQ-E1	yes	rheocene (with flow-calmed fine sedimentation)	0-1	1	05.12.2018	net	Weigand E.
8	HRQ-E2	yes	rheocene-limnocene	0-1	1	05.12.2018	net	Weigand E.
9	HRQ-Fischteich	no	ground spring	0-1	1	05.12.2018	net	Weigand E.
10	HUND	no	rheocene	0-5	1	19.10.2018	by hand	Weigand E.
11	JATA-E2	yes	rheocene	1-15	1	10.10.2018	by hand	Weigand E.
12	JÖA	yes	rheocene, very dynamic	0-15	2	29.10.2018/ 19.07.2019	net	Weigand E.

Nbr.	Spring	Monitoring spring	Type of spring	Distance from the spring outlet [m]	Nbr. of collectings	Collecting dates	Collecting method	Collector
13	KEHLS	no	N/A	0-5	1	28.03.2019	by hand	Weigand E.
14	KRAH	yes	rheocene	1-10	1	16.10.2018	by hand	Weigand E.
15	KREMS	N/A	N/A	ca. 3-15	1	21.12.2018	scooping	Fuxjäger C.
16	LILA	yes	limnocene	0-2	1	15.10.2018	by hand	Weigand E.
17	LUBO	partly	rheocene-helocene	10	1	16.11.2018	by hand	Weigand E.
18	MWGR	yes	rheocene	ca. 50	1	15.10.2018	by hand	Weigand E.
19	OCHS	N/A	rheocene	N/A	1	27.01.2020	net	Weigand E.
20	PALT	no	limnocene	0-5	1	31.10.2018	by hand	Weigand E.
21	PREBL	N/A	N/A	N/A	1	06.12.2019	net	Weigand E.
22	PUGLW	no	rheocene	N/A	1	07.11.2018	by hand	Weigand E.
23	REUT	N/A	N/A	N/A	2	21.10.2019/ 24.05.2019	net/ scooping, by hand	Weigand E./ Duda M.
24	RH-Dückenröhre	yes	N/A	subterranean	2	01.10.2012	"Triftfalle"/ scooping	Weigand E.
25	RH-Mitterberg	no	N/A	subterranean	1	01.10.2012	"Triftfalle"	Weigand E.
26	SCHA3	yes	rheocene	5-10	1	16.11.2018	by hand	Weigand E.
27	SCHÜ	yes	rheocene	1-3/1-15	2	16.11.2018/ 24.05.2019	scooping/ by hand, sieve	Weigand E./ Schubert H., Kruckenhauser L., Duda M.
28	STEY	N/A	N/A	N/A	1	31.10.2019	net	Weigand E.
29	STEYR	no	N/A	0-3	1	24.05.2019	by hand, sieve	Schubert H., Kruckenhauser L., Duda M.
30	SULZ	yes	N/A	0-4	2	23.11.2018/ 21.10.2019	net	Weigand E.
31	SULZ_B	no	N/A	0-2	1	23.11.2018	by hand	Weigand E.
32	TUFF	no	N/A	5-8	1	29.03.2019	net	Weigand E.
33	UHR1	no	rheocene	0-2	1	29.11.2018	net	Weigand E.
34	VRQ	N/A	N/A	N/A	1	31.10.2019	net	Weigand E.
35	WEIS	yes	rheocene	0-2	2	25.05.2019/ 13.11.2018	by hand, net/ sieve	Schubert H., Kruckenhauser L., Duda M./ Weigand E.
36	Welchau1+2	no	N/A	2-5/ N/A	2	23.11.2018	net/by hand	Weigand E.
37	WILD	no	N/A	0-1	1	03.04.2019	by hand, scooping	Weigand E.
38	WIND	no	rheocene	10-200	1	06.11.2018	by hand	Weigand E.
39	WOHL	no	rheocene	N/A	1	22.10.2018	by hand	Weigand E.

Supporting Table 3: Sample overview with information about taxonomy, locality, collecting date and collector

ID	Family	Genus	Species	Spring	lat	lon	Collecting date	Collector
110425/ ABOL/ 545	Bythinellidae	<i>Bythinella</i>	<i>cf. conica</i>	BRUN	47.79	14.52	17.10. 2018	Weigand E.
110425/ ABOL/ 546	Hydrobiidae	<i>Belgrandiella</i>	<i>aulaei</i>	BRUN	47.79	14.52	17.10. 2018	Weigand E.
110425/ ABOL/ 505	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	EBA-E4	47.8	14.42	08.10. 2018	Weigand E.
110425/ ABOL/ 524	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	EINS	47.9	14.37	26.10. 2018	Weigand E.
110425/ ABOL/ 483	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	GKHB	47.8	14.49	15.10. 2018	Weigand E.
110425/ ABOL/ 508	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	GÖR2	47.8	14.4	21.11. 2018	Weigand E.
110425/ ABOL/ 484	Hydrobiidae	<i>Hauffenia</i>	<i>kerschneri</i>	HRQ-E1	47.76	14.32	05.12. 2018	Weigand E.
110425/ ABOL/ 519	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	HRQ-E1	47.76	14.32	05.12. 2018	Weigand E.
110425/ ABOL/ 560	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	HRQ-E1	47.76	14.32	05.12. 2018	Weigand E.
110425/ ABOL/ 531	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	HRQ-E2	47.76	14.32	06.12. 2018	Weigand E.
110425/ ABOL/ 532	Hydrobiidae	<i>Hauffenia</i>	<i>kerschneri</i>	HRQ-E2	47.76	14.32	06.12. 2018	Weigand E.
110425/ ABOL/ 511	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	HRQ- Fischteich	47.75	14.31	05.12. 2018	Weigand E.
110425/ ABOL/ 530	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	HUND	47.74	14.42	19.10. 2018	Weigand E.
110425/ ABOL/ 507	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	JATA2	47.8	14.33	10.10. 2018	Weigand E.
110425/ ABOL/ 502	Moitessieriidae	<i>Bythiospeum</i>	<i>cf. nocki</i>	JÖA	47.78	14.43	29.10. 2018	Weigand E.
110425/ ABOL/ 503	Hydrobiidae	<i>Hauffenia</i>	<i>kerschneri</i>	JÖA	47.78	14.43	29.10. 2018	Weigand E.
110425/ ABOL/ 504	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	JÖA	47.78	14.43	29.10. 2018	Weigand E.
110425/ ABOL/ 558	Hydrobiidae	<i>Hauffenia</i>	<i>kerschneri</i>	JÖA	47.78	14.43	19.07. 2019	Weigand E.
110425/ ABOL/ 559	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	JÖA	47.78	14.43	19.07. 2019	Weigand E.
110425/ ABOL/ 510	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	KEHLS	47.75	14.34	28.03. 2019	Weigand E.
110425/ ABOL/ 536	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	KEHLS	47.75	14.34	28.03. 2019	Weigand E.
110425/ ABOL/ 515	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	KRAH	47.78	14.4	16.10. 2018	Weigand E.
110425/ ABOL/ 517	Hydrobiidae	<i>Hauffenia</i>	<i>wiener- waldensis</i>	KREMS	47.86	14.1	21.12. 2018	Fuxjäger Christian
110425/ ABOL/ 520	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	KREMS	47.86	14.1	21.12. 2018	Fuxjäger Christian
110425/ ABOL/ 509	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	LILA	47.82	14.47	15.10. 2018	Weigand E.
110425/ ABOL/ 528	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	LUBO	47.79	14.43	16.11. 2018	Weigand E.
110425/ ABOL/ 506	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	MWGR	47.8	14.49	14.10. 2018	Weigand E.
110425/ ABOL/ 567	Hydrobiidae	<i>Belgrandiella</i>	<i>aulaei</i>	OCHS	47.86	14.45	27.01. 2020	Weigand E.
110425/ ABOL/ 543	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	PALT	47.82	14.26	31.10. 2019	Weigand E.

ID	Family	Genus	Species	Spring	lat	lon	Collection date	Collector
110425/ ABOL/ 566	Hydrobiidae	<i>Belgrandiella</i>	<i>aulaei</i>	PREBL	47.89	14.31	06.12. 2019	Weigand E.
110425/ ABOL/ 523	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	PUGLW	47.68	14.48	07.11. 2018	Weigand E.
110425/ ABOL/ 525	Moitessieriidae	<i>Bythiospeum</i>	<i>cf. nocki</i>	REUT	47.82	14.37	24.05. 2019	Duda M.
110425/ ABOL/ 518	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	SCHA3	47.79	14.42	16.11. 2018	Weigand E.
110425/ ABOL/ 485	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	Schü	47.79	14.41	24.05. 2019	Schubert H., Kruckenhauser L., Duda M.
110425/ ABOL/ 513	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	SCHÜ	47.79	14.41	16.11. 2018	Weigand E.
110425/ ABOL/ 516	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	SCHÜ	47.79	14.41	16.11. 2018	Weigand E.
110425/ ABOL/ 562	Bythinellidae	<i>Bythinella</i>	<i>cf. conica</i>	STEY	47.82	14.35	21.10. 2019	Weigand E.
110425/ ABOL/ 521	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	Steyr	47.82	14.35	24.05. 2019	Schubert H., Kruckenhauser L., Duda M.
110425/ ABOL/ 522	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	Steyr	47.82	14.35	24.05. 2019	Schubert H., Kruckenhauser L., Duda M.
110425/ ABOL/ 526	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	SULZ	47.8	14.37	23.11. 2018	Weigand E.
110425/ ABOL/ 527	Moitessieriidae	<i>Bythiospeum</i>	<i>cf. nocki</i>	SULZ	47.8	14.37	23.11. 2018	Weigand E.
110425/ ABOL/ 563	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	SULZ	47.8	14.37	21.10. 2019	Weigand E.
110425/ ABOL/ 512	Hydrobiidae	<i>Hauffenia</i>	<i>kerschneri</i>	SULZ 2	47.8	14.37	23.11. 2018	Weigand E.
110425/ ABOL/ 542	Bythinellidae	<i>Bythinella</i>	<i>cf. conica</i>	SULZ 2	47.8	14.37	23.11. 2018	Weigand E.
110425/ ABOL/ 534	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	TUFF	47.72	14.56	29.03. 2019	Weigand E.
110425/ ABOL/ 544	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	UHR1	47.75	14.26	29.11. 2018	Weigand E.
110425/ ABOL/ 564	Bythinellidae	<i>Bythinella</i>	<i>cf. conica</i>	VRQ	47.79	14.2	31.10. 2019	Weigand E.
110425/ ABOL/ 565	Hydrobiidae	<i>Hauffenia</i>	<i>kerschneri</i>	VRQ	47.79	14.2	31.10. 2019	Weigand E.
110425/ ABOL/ 501	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	WEIS	47.84	14.42	25.05. 2019	Schubert H., Kruckenhauser L., Duda M.
110425/ ABOL/ 514	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	WEIS	47.84	14.42	13.11. 2018	Weigand E.
110425/ ABOL/ 547	Hydrobiidae	<i>Hauffenia</i>	<i>kerschneri</i>	Welchau 1+2	47.84	14.32	22.11. 2018	Weigand E.
110425/ ABOL/ 548	Moitessieriidae	<i>Bythiospeum</i>	<i>cf. nocki</i>	Welchau 1+2	47.84	14.32	22.11. 2018	Weigand E.
110425/ ABOL/ 549	Bythinellidae	<i>Bythinella</i>	<i>cf. conica</i>	Welchau 1+2	47.84	14.32	22.11. 2018	Weigand E.
110425/ ABOL/ 540	Bythinellidae	<i>Bythinella</i>	<i>cf. conica</i>	Welchau1	47.84	14.32	22.11. 2018	Weigand E.
110425/ ABOL/ 535	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	WILD	47.82	14.43	03.04. 2019	Weigand E.
110425/ ABOL/ 539	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	WILD	47.82	14.43	03.04. 2019	Weigand E.
110425/ ABOL/ 529	Bythinellidae	<i>Bythinella</i>	<i>conica</i>	WIND	47.82	14.2	06.11. 2018	Weigand E.

ID	Family	Genus	Species	Spring	lat	lon	Collection date	Collector
110425/ ABOL/ 541	Bythinellidae	<i>Bythinella</i>	<i>cf. conica</i>	Wohl	47.73	14.44	22.10. 2019	Weigand E.

Supporting Table 4: Specimen overview with DNA concentration of both eluates, BOLD number, PCR and Sequencing primer. Conc.=concentration, E=eluate

ID	BOLD numbers	Genus	Species	DNA Conc. [ng/μ] E1	DNA Conc. [ng/μ] E2	PCR primer	Sequencing primer
ABOL_483_1	AMOL577-20	<i>Bythinella</i>	<i>conica</i>	21.7	6.36	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_483_2	AMOL578-20	<i>Bythinella</i>	<i>conica</i>	23.5	9.01	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_483_3	AMOL579-20	<i>Bythinella</i>	<i>conica</i>	46.4	27.4	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_484_1	AMOL580-20	<i>Hauffenia</i>	<i>kerschneri</i>	5.24	1.55	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_485_1	AMOL581-20	<i>Bythinella</i>	<i>conica</i>	14.4	2.55	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_485_2	AMOL582-20	<i>Bythinella</i>	<i>conica</i>	16.4	4.64	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_485_3	AMOL583-20	<i>Bythinella</i>	<i>conica</i>	31.3	25.3	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_501_1	AMOL584-20	<i>Bythinella</i>	<i>conica</i>	22.3	8.65	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_501_2	AMOL585-20	<i>Bythinella</i>	<i>conica</i>	29.3	14.8	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_501_3	AMOL586-20	<i>Bythinella</i>	<i>conica</i>	13.3	8.44	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_502		<i>Bythiospeum</i>	<i>cf.nocki</i>	too low	too low	-	-
ABOL_503_1	AMOL587-20	<i>Hauffenia</i>	<i>kerschneri</i>	5.56	3.91	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_503_2	AMOL588-20	<i>Hauffenia</i>	<i>kerschneri</i>	3.68	1.72	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_503_3	AMOL589-20	<i>Hauffenia</i>	<i>kerschneri</i>	3.89	2.31	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_504_1	AMOL590-20	<i>Bythinella</i>	<i>conica</i>	14	4.57	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3

ID	BOLD numbers	Genus	Species	DNA Conc. [ng/μ] E1	DNA Conc. [ng/μ] E2	PCR primer	Sequencing primer
ABOL_504_2		<i>Bythinella</i>	<i>conica</i>	1.37	0.567	LCO1490_Hydrob1/ HCO2216_Hydrob3	-
ABOL_504_3	AMOL591-20	<i>Bythinella</i>	<i>conica</i>	20.5	5.4	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_505_1	AMOL592-20	<i>Bythinella</i>	<i>conica</i>	43.5	17	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_505_2	AMOL593-20	<i>Bythinella</i>	<i>conica</i>	36.7	17.6	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_505_3	AMOL594-20	<i>Bythinella</i>	<i>conica</i>	39.6	16.6	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_506_1	AMOL595-20	<i>Bythinella</i>	<i>conica</i>	25.1	13	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_506_2	AMOL596-20	<i>Bythinella</i>	<i>conica</i>	16.1	7.8	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_506_3	AMOL597-20	<i>Bythinella</i>	<i>conica</i>	18.7	8.16	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_507_1	AMOL598-20	<i>Bythinella</i>	<i>conica</i>	18.9	9.89	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_507_2	AMOL599-20	<i>Bythinella</i>	<i>conica</i>	14.3	8.31	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_507_3	AMOL600-20	<i>Bythinella</i>	<i>conica</i>	23.7	15.6	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_508_1	AMOL601-20	<i>Bythinella</i>	<i>conica</i>	22.7	6.42	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_508_2	AMOL602-20	<i>Bythinella</i>	<i>conica</i>	24.7	7.37	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_508_3	AMOL603-20	<i>Bythinella</i>	<i>conica</i>	21.1	4.12	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_509_1	AMOL604-20	<i>Bythinella</i>	<i>conica</i>	20.5	10.1	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro

ID	BOLD numbers	Genus	Species	DNA Conc. [ng/μ] E1	DNA Conc. [ng/μ] E2	PCR primer	Sequencing primer
ABOL_509_2	AMOL605-20	<i>Bythinella</i>	<i>conica</i>	16.7	8.62	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_509_2	AMOL605-20	<i>Bythinella</i>	<i>conica</i>	16.7	8.62	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_509_3	AMOL606-20	<i>Bythinella</i>	<i>conica</i>	17	9.36	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_510_1	AMOL607-20	<i>Bythinella</i>	<i>conica</i>	46.2	15.9	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_510_2	AMOL608-20	<i>Bythinella</i>	<i>conica</i>	19.6	3.24	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_510_3	AMOL609-20	<i>Bythinella</i>	<i>conica</i>	19.3	9.68	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_510_5	AMOL610-20	<i>Bythinella</i>	<i>conica</i>	15.3	4.87	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_510_6	AMOL611-20	<i>Bythinella</i>	<i>conica</i>	13.4	1.83	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_510_7	AMOL612-20	<i>Bythinella</i>	<i>conica</i>	18.3	4.21	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_511	AMOL613-20	<i>Bythinella</i>	<i>conica</i>	0.30 3	0.093	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_512_1	AMOL614-20	<i>Hauffenia</i>	<i>kerschneri</i>	5.2	1.71	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_513	AMOL615-20	<i>Bythinella</i>	<i>conica</i>	0.08	too low	LCO1490_Hydrob1/ HCO2198_Hydrob1	LCO1490_Hydrob1/ HCO2198_Hydrob1
ABOL_514_1	AMOL616-20	<i>Bythinella</i>	<i>conica</i>	too low	0.119	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_514_2	AMOL617-20	<i>Bythinella</i>	<i>conica</i>	1.85	0.437	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_514_3	AMOL618-20	<i>Bythinella</i>	<i>conica</i>	1.16	0.472	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_515_1	AMOL619-20	<i>Bythinella</i>	<i>conica</i>	16.2	5.07	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro

ID	BOLD numbers	Genus	Species	DNA Conc. [ng/μl] E1	DNA Conc. [ng/μl] E2	PCR primer	Sequencing primer
ABOL_515_2	AMOL620-20	<i>Bythinella</i>	<i>conica</i>	22.4	5.95	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_515_2	AMOL620-20	<i>Bythinella</i>	<i>conica</i>	22.4	5.95	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_515_3	AMOL621-20	<i>Bythinella</i>	<i>conica</i>	20.2	8.07	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_516_1	AMOL622-20	<i>Bythinella</i>	<i>conica</i>	2.26	too low	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_516_2	AMOL623-20	<i>Bythinella</i>	<i>conica</i>	4.61	0.926	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_516_3	AMOL624-20	<i>Bythinella</i>	<i>conica</i>	2.95	0.48	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_517_1	AMOL625-20	<i>Hauffenia</i>	<i>wiener-waldensis</i>	5.45	3.69	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_517_2	AMOL626-20	<i>Hauffenia</i>	<i>wiener-waldensis</i>	2.16	2.8	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_517_3	AMOL627-20	<i>Hauffenia</i>	<i>wiener-waldensis</i>	3.07	2.91	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_518_1	AMOL628-20	<i>Bythinella</i>	<i>conica</i>	33.9	21	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_518_2	AMOL629-20	<i>Bythinella</i>	<i>conica</i>	20	12.8	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_518_3	AMOL630-20	<i>Bythinella</i>	<i>conica</i>	38	22.3	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_519_1	AMOL631-20	<i>Bythinella</i>	<i>conica</i>	28	12.4	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_519_2	AMOL632-20	<i>Bythinella</i>	<i>conica</i>	35.5	16.9	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_519_3	AMOL633-20	<i>Bythinella</i>	<i>conica</i>	33.5	12.6	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro	LCO1490_ABOL_Moll_1/ HCO2216_ABOL_Moll_3_pro
ABOL_520_1	AMOL634-20	<i>Bythinella</i>	<i>conica</i>	9.26	7.57	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_520_2	AMOL635-20	<i>Bythinella</i>	<i>conica</i>	16.5	5.46	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3

ID	BOLD numbers	Genus	Species	DNA Conc. [ng/μl] E1	DNA Conc. [ng/μl] E2	PCR primer	Sequencing primer
ABOL_520_3	AMOL636-20	<i>Bythinella</i>	<i>conica</i>	12.4	4.41	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_521	AMOL637-20	<i>Bythinella</i>	<i>conica</i>	6.27	2.9	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_522_1	AMOL638-20	<i>Bythinella</i>	<i>conica</i>	23.4	14.3	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_522_2	AMOL639-20	<i>Bythinella</i>	<i>conica</i>	21.7	8.22	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_522_3	AMOL640-20	<i>Bythinella</i>	<i>conica</i>	32.5	28.9	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro	LCO1490_ABOL_ Moll_1/ HCO2216_ABOL_ Moll_3_pro
ABOL_523_1	AMOL641-20	<i>Bythinella</i>	<i>conica</i>	26.9	14.3	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_523_2	AMOL642-20	<i>Bythinella</i>	<i>conica</i>	28.3	17.7	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_523_3	AMOL643-20	<i>Bythinella</i>	<i>conica</i>	48.8	48.6	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_524_1	AMOL644-20	<i>Bythinella</i>	<i>conica</i>	44.5	37.5	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_524_2	AMOL645-20	<i>Bythinella</i>	<i>conica</i>	20.8	5.54	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_524_3	AMOL646-20	<i>Bythinella</i>	<i>conica</i>	14.5	7.05	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_525		<i>Bythiospeum</i>	<i>cf. nocki</i>			-	-
ABOL_526_1	AMOL647-20	<i>Bythinella</i>	<i>conica</i>	23.2	8.4	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_526_2	AMOL648-20	<i>Bythinella</i>	<i>conica</i>	10.6	2.63	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_526_3	AMOL649-20	<i>Bythinella</i>	<i>conica</i>	12	2.94	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_527		<i>Bythiospeum</i>	<i>cf. nocki</i>	too low	too low	-	-
ABOL_528_1	AMOL650-20	<i>Bythinella</i>	<i>conica</i>	54	26	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_528_2	AMOL651-20	<i>Bythinella</i>	<i>conica</i>	42.4	27.9	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_528_3	AMOL652-20	<i>Bythinella</i>	<i>conica</i>	47.5	27.5	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_529_1	AMOL653-20	<i>Bythinella</i>	<i>conica</i>	30.7	13.9	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3

ID	BOLD numbers	Genus	Species	DNA Conc. [ng/μ] E1	DNA Conc. [ng/μ] E2	PCR primer	Sequencing primer
ABOL_529_2	AMOL654-20	<i>Bythinella</i>	<i>conica</i>	33.8	18.5	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_529_3	AMOL655-20	<i>Bythinella</i>	<i>conica</i>	41.7	21.6	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_530_1	AMOL656-20	<i>Bythinella</i>	<i>conica</i>	18.6	8.17	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_530_2	AMOL657-20	<i>Bythinella</i>	<i>conica</i>	36.2	13.8	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_530_3	AMOL658-20	<i>Bythinella</i>	<i>conica</i>	11.6	4.86	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_531_1	AMOL659-20	<i>Bythinella</i>	<i>conica</i>	27.8	11.1	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_531_2	AMOL660-20	<i>Bythinella</i>	<i>conica</i>	17.3	7.44	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_531_3	AMOL661-20	<i>Bythinella</i>	<i>conica</i>	19.6	6.39	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_532_1	AMOL662-20	<i>Hauffenia</i>	<i>kerschneri</i>	3.08	1.78	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_534_1	AMOL663-20	<i>Bythinella</i>	<i>conica</i>	24.9	5.11	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_534_2	AMOL664-20	<i>Bythinella</i>	<i>conica</i>	7.32	2.22	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_534_3	AMOL665-20	<i>Bythinella</i>	<i>conica</i>	15.4	4.04	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_535		<i>Bythinella</i>	<i>conica</i>			-	-
ABOL_536_1	AMOL666-20	<i>Bythinella</i>	<i>conica</i>	2.32	0.417	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_539_1	AMOL667-20	<i>Bythinella</i>	<i>conica</i>	2.87	0.377	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_540		<i>Bythinella</i>	<i>cf. conica</i>			-	-
ABOL_541		<i>Bythinella</i>	<i>cf. conica</i>			-	-
ABOL_542		<i>Bythinella</i>	<i>cf. conica</i>			-	-
ABOL_543_1	AMOL668-20	<i>Bythinella</i>	<i>conica</i>	15	4.13	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_543_2	AMOL669-20	<i>Bythinella</i>	<i>conica</i>	25.4	5.8	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_543_3	AMOL670-20	<i>Bythinella</i>	<i>conica</i>	21.2	3.96	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_544_1	AMOL671-20	<i>Bythinella</i>	<i>conica</i>	32.9	15.4	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_544_2	AMOL672-20	<i>Bythinella</i>	<i>conica</i>	19.9	5.13	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3

ID	BOLD numbers	Genus	Species	DNA Conc. [ng/μ] E1	DNA Conc. [ng/μ] E2	PCR primer	Sequencing primer
ABOL_544_3	AMOL673-20	<i>Bythinella</i>	<i>conica</i>	20.3	6.77	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_545		<i>Bythinella</i>	<i>cf. conica</i>			-	-
ABOL_546_1	AMOL674-20	<i>Belgrandiella</i>	<i>aulaei</i>	3.14	2.06	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_546_2	AMOL675-20	<i>Belgrandiella</i>	<i>aulaei</i>	3.2	1.49	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_546_3	AMOL676-20	<i>Belgrandiella</i>	<i>aulaei</i>	2.87	1.73	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_547_1	AMOL677-20	<i>Hauffenia</i>	<i>kerschneri</i>	6.17	2.91	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_548		<i>Bythiospeum</i>	<i>cf. nocki</i>	too low	too low	-	-
ABOL_549		<i>Bythinella</i>	<i>cf. conica</i>			-	-
ABOL_558		<i>Hauffenia</i>	<i>kerschneri</i>			-	-
ABOL_559		<i>Bythinella</i>	<i>conica</i>			-	-
ABOL_560	AMOL678-20	<i>Bythinella</i>	<i>conica</i>	2.85	1.78	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_562		<i>Bythinella</i>	<i>cf. conica</i>			-	-
ABOL_563		<i>Bythinella</i>	<i>conica</i>			-	-
ABOL_564		<i>Bythinella</i>	<i>cf. conica</i>			-	-
ABOL_565_1	AMOL720-20	<i>Hauffenia</i>	<i>kerschneri</i>	3.33	1.21	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_566_1	AMOL721-20	<i>Belgrandiella</i>	<i>aulaei</i>	4.3	1.69	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_567_1	AMOL722-20	<i>Belgrandiella</i>	<i>aulaei</i>	5.16	1.77	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_567_2	AMOL723-20	<i>Belgrandiella</i>	<i>aulaei</i>	1.53	1.05	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3
ABOL_567_3	AMOL724-20	<i>Belgrandiella</i>	<i>aulaei</i>	2.58	1.33	LCO1490_Hydrob1/ HCO2216_Hydrob3	LCO1490_Hydrob1/ HCO2216_Hydrob3

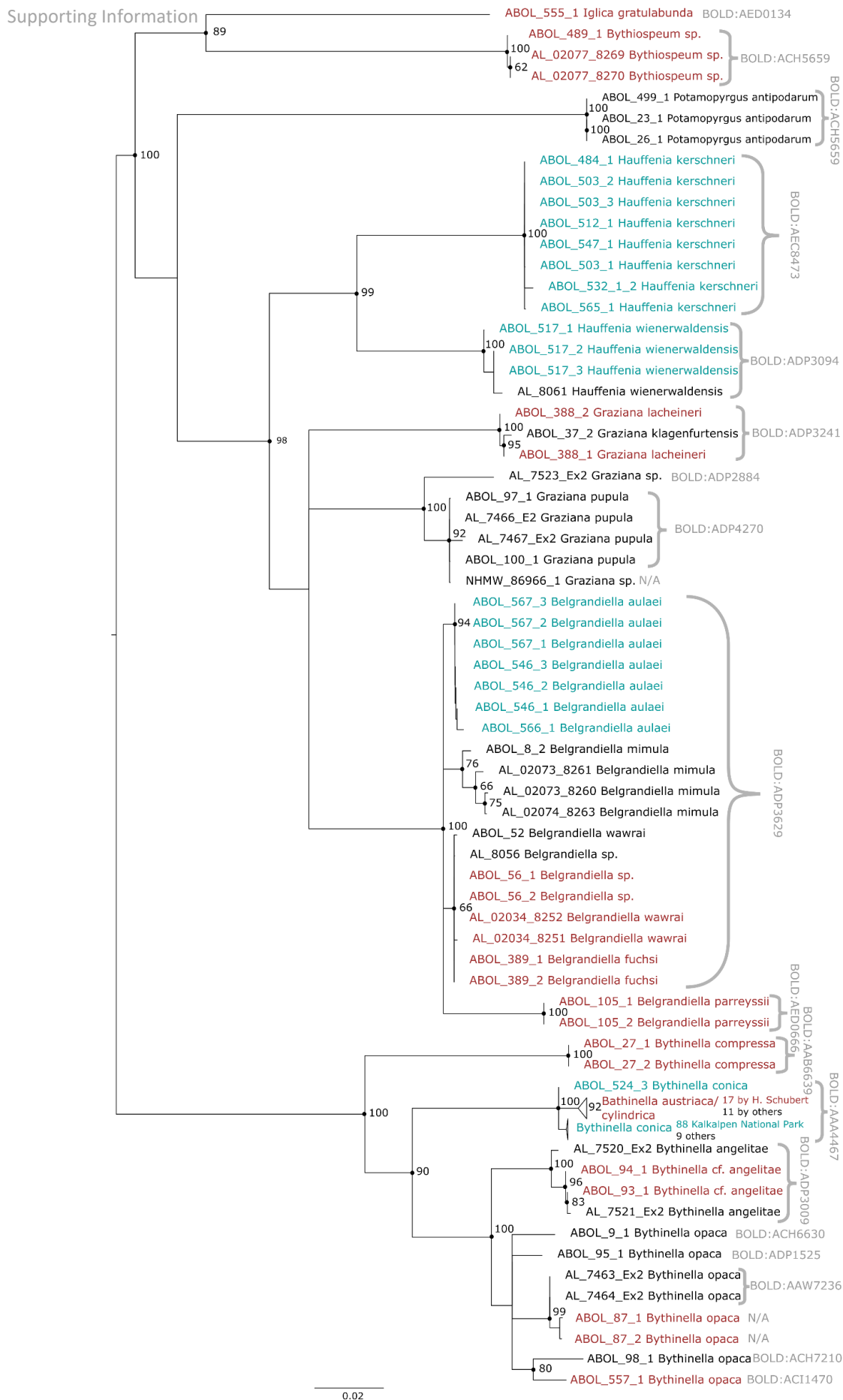
Supporting Table 5: Specimen overview of hydrobioid sequences from the ABOL Mollusca project used for comparison. The table includes information about BOLD number, BIN affiliation, locality and collecting date (as far as available). DNA barcodes created in the course of this study are marked with an asterisk. Bgld=Burgenland, DE=Deutschland, NÖ=Niederösterreich, OÖ=Oberösterreich, Sbg=Salzburg, Stmk=Steiermark, Ktn=Kärnten, W=Wien

ID	BOLD nrs.	BIN BOLD	Species	State	Locality	lat	lon	Collecting date	Collector
ABOL_105_1*	AMOL 713-20	BOLD: AED0666	Belgrandiella parreyssii	NÖ	Bad Vöslau	47.97	16.22	14.08. 2014	Mrkvicka A.
ABOL_105_2*	AMOL 714-20	BOLD: AED0666	Belgrandiella parreyssii	NÖ	Bad Vöslau	47.97	16.22	14.08. 2014	Mrkvicka A.
ABOL_389_1*	AMOL 717-20	BOLD: ADP3629	Belgrandiella fuchsi	NÖ	Niederösterreichische Kalkalpen	47.98	15.74	24.07. 2018	Mrkvicka A.
ABOL_389_2*	AMOL 718-20	BOLD: ADP3629	Belgrandiella fuchsi	NÖ	Niederösterreichische Kalkalpen	47.98	15.74	24.07. 2018	Mrkvicka A.
ABOL_52_1	AMOL 037-18	BOLD: ADP3629	Belgrandiella wawrai	NÖ	Niederösterreichische Kalkalpen	47.98	15.91	12.10. 2012	Mrkvicka A.
ABOL_56_1*	AMOL 709-20	BOLD: ADP3629	Belgrandiella sp.	NÖ	Niederösterreichische Kalkalpen	48.00	15.93	13.10. 2012	Mrkvicka A.
ABOL_56_2*	AMOL 710-20	BOLD: ADP3629	Belgrandiella sp.	NÖ	Niederösterreichische Kalkalpen	48.00	15.93	13.10. 2012	Mrkvicka A.
ABOL_8_1	AMOL 048-18	BOLD: ADP3629	Belgrandiella mimula	NÖ	Bad Fischau	47.83	16.18	2009	Mrkvicka A.
ABOL_8_2	AMOL 049-18	BOLD: ADP3629	Belgrandiella mimula	NÖ	Bad Fischau	47.83	16.18	2009	Mrkvicka A.
AL_8056	AMOL 568-18	BOLD: ADP3629	Belgrandiella sp.	NÖ	Niederösterreichische Kalkalpen	48.00	15.93	27.10. 2016	Feher Z., Grego J.
AL_8251*	AMOL 685-20	BOLD: ADP3629	Belgrandiella wawrai	NÖ	Niederösterreichische Kalkalpen	47.99	15.90	13.06. 2017	Duda M.
AL_8252*	AMOL 686-20	BOLD: ADP3629	Belgrandiella wawrai	NÖ	Niederösterreichische Kalkalpen	47.99	15.90	13.06. 2017	Duda M.
AL_8260	AMOL 688-20	BOLD: ADP3629	Belgrandiella mimula	NÖ	Bad Fischau	47.83	16.17	20.11. 2019	Duda M., Kruckenhauser L., Lajtner J.
AL_8261	AMOL 689-20	BOLD: ADP3629	Belgrandiella mimula	NÖ	Bad Fischau	47.83	16.17	20.11. 2019	Duda M., Kruckenhauser L., Lajtner J.
AL_8263	AMOL 690-20	BOLD: ADP3629	Belgrandiella mimula	NÖ	Bad Fischau	47.83	16.17	20.11. 2019	Duda M., Kruckenhauser L., Lajtner J.
ABOL_108_1*	AMOL 700-20	BOLD: AAA4467	Bythinella conica	OÖ	Salzkammergut	47.89	13.78	26.07. 2015	Steger J., Mähner B.
ABOL_21_1*	AMOL 687-20	BOLD: AAA4467	Bythinella conica	Stmk	Altaussee			2009	Mrkvicka A.
ABOL_27_1*	AMOL 707-20	BOLD: AAB6639	Bythinella compressa	DE	Rhön			13.08. 2009	Mrkvicka A.
ABOL_27_2*	AMOL 708-20	BOLD: AAB6639	Bythinella compressa	DE	Rhön			13.08. 2009	Mrkvicka A.

ID	BOLD nbrs.	BIN BOLD	Species	State	Locality	lat	lon	Collec- tion date	Collector
ABOL_419_1*	AMOL 679-20	BOLD: AAA4467	Bythinella austriaca	Bgld	Großhöflein, Radegundisbründl	47.84	16.48	01.06. 2017	Moog & Christian
ABOL_419_2*	AMOL 680-20	BOLD: AAA4467	Bythinella austriaca	Bgld	Großhöflein, Radegundisbründl	47.84	16.48	01.06. 2017	Moog & Christian
ABOL_425_1*	AMOL 681-20	BOLD: AAA4467	Bythinella austriaca	NÖ	Heiligenkreuz, Sagbründl	48.06	16.13	01.06. 2017	Moog & Christian
ABOL_425_2*	AMOL 682-20	BOLD: AAA4467	Bythinella austriaca	NÖ	Heiligenkreuz, Sagbründl	48.06	16.13	01.06. 2017	Moog & Christian
ABOL_425_4*	AMOL 683-20	BOLD: AAA4467	Bythinella austriaca	NÖ	Heiligenkreuz, Sagbründl	48.06	16.13	01.06. 2017	Moog & Christian
ABOL_425_5*	AMOL 684-20	BOLD: AAA4467	Bythinella austriaca	NÖ	Heiligenkreuz, Sagbründl	48.06	16.13	01.06. 2017	Moog & Christian
ABOL_43_1*	AMOL 030-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Adlitzgräben			2012	Mrkvicka A.
ABOL_46_1	AMOL 031-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Further Tal	47.99	15.89	12.10. 2012	Mrkvicka A.
ABOL_550_1*	AMOL 701-20	BOLD: AAA4467	Bythinella austriaca	Stmk	Frein/Mürz	47.73	15.49	10.11. 2019	Plan L.
ABOL_556_1*	AMOL 703-20	BOLD: AAA4467	Bythinella austriaca	NÖ	Schwarzau im Gebirge	47.81	15.71	11.11. 2018	Mrkvicka A.
ABOL_557_1*	AMOL 704-20	BOLD: AC1470	Bythinella opaca	Ktn	GarlFal	46.70	12.96	01.06. 2019	Mrkvicka A.
ABOL_57_1	AMOL 039-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Laabachtal	48.01	15.88	13.10. 2012	Mrkvicka A.
ABOL_62_1	AMOL 041-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Höfnergraben	48.00	15.93	13.10. 2012	Mrkvicka A.
ABOL_64_1	AMOL 042-18	BOLD: AAA4467	Bythinella conica	OÖ	Molln	47.91	14.24	22.06. 2013	Mrkvicka A.
ABOL_64_2	AMOL 043-18	BOLD: AAA4467	Bythinella conica	OÖ	Molln	47.91	14.24	22.06. 2013	Mrkvicka A.
ABOL_66_1*	AMOL 705-20	BOLD: AAA4467	Bythinella austriaca	NÖ	ober Neuwaldegg	48.25	16.25	27.06. 2013	Mrkvicka A.
ABOL_66_2*	AMOL 706-20	BOLD: AAA4467	Bythinella austriaca	NÖ	ober Neuwaldegg	48.25	16.25	27.06. 2013	Mrkvicka A.
ABOL_9_1	AMOL 051-18	BOLD: ACH6630	Bythinella opaca	Stmk	Zeutschach			2009	Mrkvicka A.
ABOL_93_1*	AMOL 711-20	BOLD: ADP3009	Bythinella cf. angelitae	Ktn	Klagenfurt-Land, Loibltal	46.47	14.26	18.07. 2014	Mrkvicka A.
ABOL_94_1*	AMOL 712-20	BOLD: ADP3009	Bythinella cf. angelitae	Ktn	Klagenfurt-Land, Loibltal	46.47	14.26	18.07. 2014	Mrkvicka A.
ABOL_95_1	AMOL 052-18	BOLD: ADP1525	Bythinella opaca	Ktn	Keutschach	46.58	14.12	23.07. 2014	Mrkvicka A.
ABOL_98_1	AMOL 054-18	BOLD: ACH7210	Bythinella opaca	Ktn	Reifnitz			16.07. 2014	Mrkvicka A.

ID	BOLD nbrs.	BIN BOLD	Species	State	Locality	lat	lon	Collec- tion date	Collector
AL_ 7442	AMOL 177-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Wienerwald- Pfalzberg	48.16	16.04	21.04. 2015	Duda M.
AL_ 7444	AMOL 178-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Wienerwald- Pfalzberg	48.17	16.06	21.04. 2015	Duda M.
AL_ 7463	AMOL 192-18	BOLD: AAW7236	Bythinella opaca	Ktn	Karawanken- Remschenig	46.46	14.62	29.06. 2015	Duda M. and others
AL_ 7464	AMOL 193-18	BOLD: AAW7236	Bythinella opaca	Ktn	Karawanken- Remschenig	46.46	14.62	29.06. 2015	Duda M. and others
AL_ 7520	AMOL 219-18	BOLD: ADP3009	Bythinella angelitae	Ktn	Karawanken- Tscheppa- schlucht	46.49	14.28	01.07. 2015	Duda M. and others
AL_ 7521	AMOL 220-18	BOLD: ADP3009	Bythinella angelitae	Ktn	Karawanken- Tscheppa- schlucht	46.49	14.28	01.07. 2015	Duda M. and others
AL_ 7664	AMOL 261-18	BOLD: AAA4467	Bythinella conica	NÖ	Scheibbs- Dürrenstein	47.76	15.00	11.08. 2015	Duda M. and others
AL_ 7982	AMOL 414-18	BOLD: AAA4467	Bythinella conica	NÖ	Dürrenstein- Hochrzeith	47.77	14.96	26.06. 2016	Duda M., Bamberger S., Fischer S.
AL_ 7984	AMOL 415-18	BOLD: AAA4467	Bythinella conica	NÖ	Scheibbs- Dürrenstein	47.76	15.00	23.05. 2015	Fischer S.
AL_ 7987	AMOL 416-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Scheibbs- Dürrenstein	47.78	15.11	28.05. 2014	Colling M.
AL_ 7988	AMOL 417-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Scheibbs- Dürrenstein	47.78	15.11	28.05. 2014	Colling M.
AL_ 7991	AMOL 418-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Scheibbs- Dürrenstein	47.78	15.09	29.07. 2014	Colling M.
AL_ 7995	AMOL 419-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Scheibbs- Dürrenstein	47.77	15.07	30.07. 2014	Colling M.
AL_ 8272*	AMOL 693-20	BOLD: AAA4467	Bythinella austriaca	NÖ	Wienerwald	48.25	16.25	29.10. 2019	Duda M., Mrkvicka A., Schubert H.
AL_ 8275*	AMOL 694-20	BOLD: AAA4467	Bythinella cylindrica	NÖ	Triestingtal	47.97	16.08	01.03. 2014	Reischütz A.
AL_ 8276*	AMOL 695-20	BOLD: AAA4467	Bythinella cylindrica	NÖ	Triestingtal	47.97	16.08	01.03. 2014	Reischütz A.
AL_ 8278*	AMOL 696-20	BOLD: AAA4467	Bythinella austriaca	NÖ	Triestingtal	47.97	16.08	01.03. 2014	Reischütz A.
AL_ 8279*	AMOL 697-20	BOLD: AAA4467	Bythinella austriaca	NÖ	Triestingtal	47.97	16.08	01.03. 2014	Reischütz A.
AL_ 8284*	AMOL 698-20	BOLD: AAA4467	Bythinella cylindrica	NÖ	Pottenstein	47.97	16.07	01.05. 2019	Reischütz A.
AL_ 8285*	AMOL 699-20	BOLD: AAA4467	Bythinella cylindrica	NÖ	Pottenstein	47.97	16.07	01.05. 2019	Reischütz A.
HNSM_ 108421	AMOL 490-18	BOLD: AAA4467	Bythinella conica	Sbg	Pongau	47.43	13.28	06.09. 2011	Boeters H.
HNSM_ 122541	AMOL 492-18	BOLD: AAA4467	Bythinella conica	Sbg	Flachgau	47.80	13.29	10.07. 2012	Boeters H.

ID	BOLD nbrs.	BIN BOLD	Species	State	Locality	lat	lon	Collection date	Collector
ABOL_489_1*	AMOL 719-20	BOLD: ACH5659	Bythiospeum sp.	W	Lainzer Tiergarten				Mrkvicka A.
AL_8269*	AMOL 691-20	BOLD: ACH5659	Bythiospeum sp.	W	Wienerwald-Lainzer Tiergarten	48.18	16.23	29.10. 2019	Duda M., Mrkvicka A., Schubert H.
AL_8270*	AMOL 692-20	BOLD: ACH5659	Bythiospeum sp.	W	Wienerwald-Lainzer Tiergarten	48.18	16.23	29.10. 2019	Duda M., Mrkvicka A., Schubert H.
ABOL_100_1	AMOL 003-18	BOLD: ADP4270	Graziana pupula	Ktn	Keutschach	46.58	14.23	22.07. 2014	Mrkvicka A.
ABOL_37_2	AMOL 028-18	BOLD: ADP3241	Graziana klagenfurtensis	Ktn	Klagenfurt			2011	Mrkvicka A.
ABOL_388_1*	AMOL 715-20	BOLD: ADP3241	Graziana lacheineri	Ktn	St. Paul im Lavanttal	46.69	14.87	19.08. 2018	Mrkvicka A.
ABOL_388_2*	AMOL 716-20	BOLD: ADP3241	Graziana lacheineri	Ktn	St. Paul im Lavanttal	46.69	14.87	19.08. 2018	Mrkvicka A.
ABOL_97_1	AMOL 053-18	BOLD: ADP4270	Graziana pupula	Ktn	Reifnitz	46.60	14.17	16.07. 2014	Mrkvicka A.
AL_7466	AMOL 194-18	BOLD: ADP4270	Graziana pupula	Ktn	Karawanken-Remschenig	46.46	14.61	29.06. 2015	Duda M. and others
AL_7467	AMOL 195-18	BOLD: ADP4270	Graziana pupula	Ktn	Karawanken-Remschenig	46.46	14.61	29.06. 2015	Duda M. and others
AL_7523	AMOL 221-18	BOLD: ADP2884	Graziana sp.	Ktn	Karawanken-Tscheppaschlucht	46.49	14.28	01.07. 2015	Duda M. and others
AL_8061	AMOL 456-18	BOLD: ADP3094	Hauffenia wienerwaldensis	W	Döbling	48.27	16.32	11.04. 2017	Moog O.
ABOL_555_1*	AMOL 702-20	BOLD: AED0134	Iglica gratulabunda	Stmk	Kapfenberg	47.44	15.29	25.06. 2019	Boeters, Reischütz A., Unruh
ABOL_23_1	AMOL 022-18	BOLD: ABY5556	Potamopyrgus antipodarum	Bgld	Leithaprodersdorf			2009	Mrkvicka A.
ABOL_26_1	AMOL 023-18	BOLD: ABY5556	Potamopyrgus antipodarum	NÖ	Pfaffstätten			2009	Mrkvicka A.
ABOL_499_1	AMOL 036-18	BOLD: ABY5556	Potamopyrgus antipodarum	NÖ	Perchtoldsdorf			09.09. 2016	Mrkvicka A.
NHMW-86966_1			Graziana sp.	NÖ	Haschendorf			23.03. 2017	Haase M.
ABOL_55_1	AMOL 038-18	BOLD: AAA4467	Bythinella austriaca	NÖ	Further Tal	47.98	15.92	12.10. 2012	Mrkvicka A.
ABOL_87_1*			Bythinella opaca	Ktn				05.04. 2014	Mrkvicka A.
ABOL_87_2*			Bythinella opaca	Ktn				05.04. 2014	Mrkvicka A.



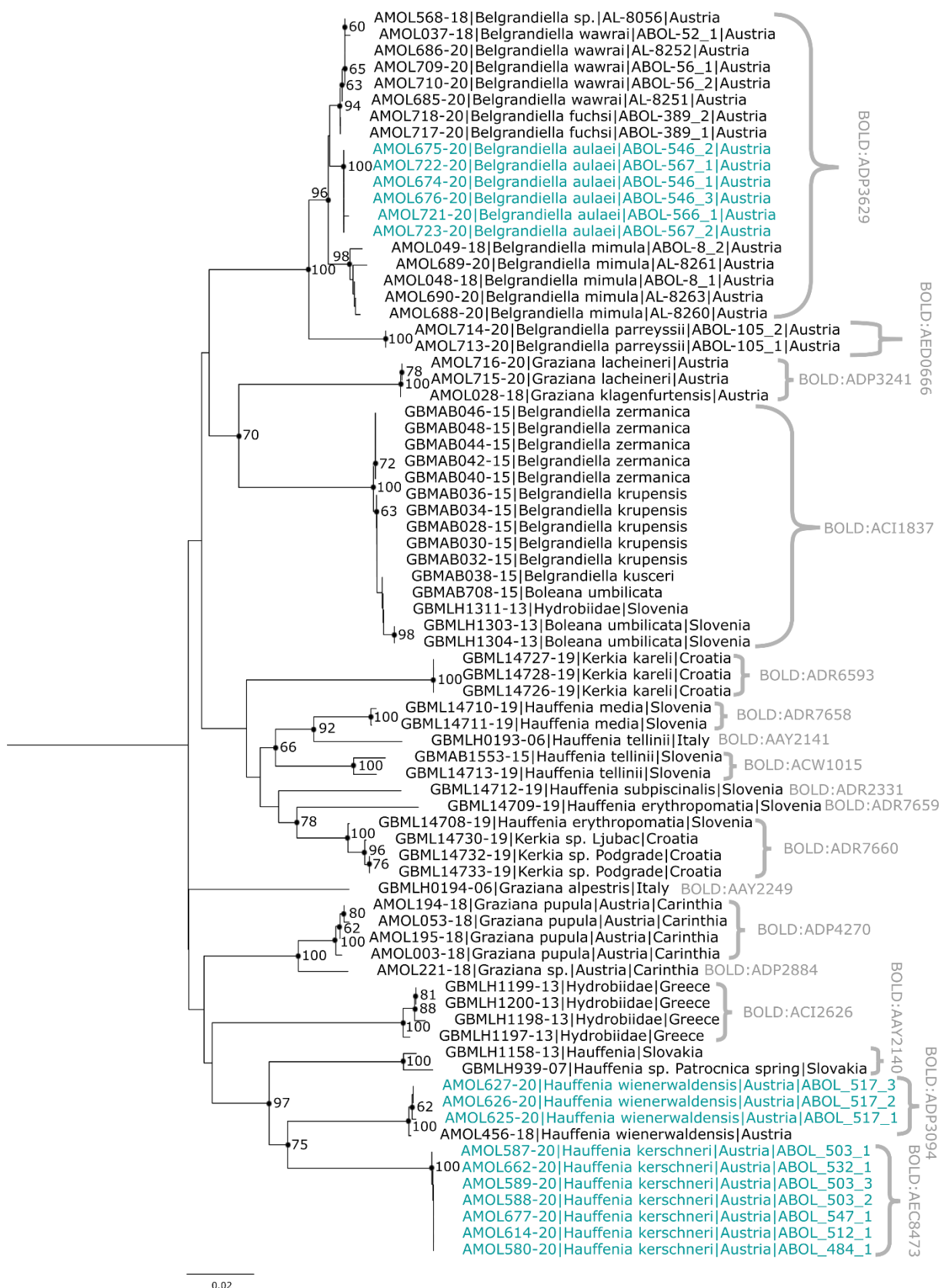
Supporting Figure 1: Neighbor joining tree with supporting bootstrap values (only values above 60 are displayed here) of all hydrobioid sequences from this study and the ABOL Mollusca project. Blue: Sequences from specimen of the Kalkalpen National Park and its surroundings. Red: Sequences made in the course of this study by H. Schubert for the ABOL Mollusca project. Grey: BIN numbers

Supporting Table 6: Specimen overview of hydrobioid sequences from the Kalkalpen National Park and its surrounding, that were uploaded to BOLD, including information about BOLD ID and BIN Affiliation.

ID	BOLD numbers	BIN BOLD	Genus	Species
ABOL_483_1	AMOL577-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_483_2	AMOL578-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_483_3	AMOL579-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_484_1	AMOL580-20	BOLD:AEC8473	<i>Hauffenia</i>	<i>kerschneri</i>
ABOL_485_1	AMOL581-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_485_2	AMOL582-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_485_3	AMOL583-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_501_1	AMOL584-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_501_2	AMOL585-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_501_3	AMOL586-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_503_1	AMOL587-20	BOLD:AEC8473	<i>Hauffenia</i>	<i>kerschneri</i>
ABOL_503_2	AMOL588-20	BOLD:AEC8473	<i>Hauffenia</i>	<i>kerschneri</i>
ABOL_503_3	AMOL589-20	BOLD:AEC8473	<i>Hauffenia</i>	<i>kerschneri</i>
ABOL_504_1	AMOL590-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_504_3	AMOL591-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_505_1	AMOL592-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_505_2	AMOL593-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_505_3	AMOL594-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_506_1	AMOL595-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_506_2	AMOL596-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_506_3	AMOL597-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_507_1	AMOL598-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_507_2	AMOL599-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_507_3	AMOL600-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_508_1	AMOL601-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_508_2	AMOL602-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_508_3	AMOL603-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_509_1	AMOL604-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_509_2	AMOL605-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_509_3	AMOL606-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_510_1	AMOL607-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_510_2	AMOL608-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_510_3	AMOL609-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_510_5	AMOL610-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_510_6	AMOL611-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_510_7	AMOL612-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_511	AMOL613-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_512_1	AMOL614-20	BOLD:AEC8473	<i>Hauffenia</i>	<i>kerschneri</i>
ABOL_513	AMOL615-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_514_1	AMOL616-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_514_2	AMOL617-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_514_3	AMOL618-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_515_1	AMOL619-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_515_2	AMOL620-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>

ID	BOLD numbers	BIN BOLD	Genus	Species
ABOL_515_3	AMOL621-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_516_1	AMOL622-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_516_2	AMOL623-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_516_3	AMOL624-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_517_1	AMOL625-20	BOLD:ADP3094	<i>Hauffenia</i>	<i>wienerwaldensis</i>
ABOL_517_2	AMOL626-20	BOLD:ADP3094	<i>Hauffenia</i>	<i>wienerwaldensis</i>
ABOL_517_3	AMOL627-20	BOLD:ADP3094	<i>Hauffenia</i>	<i>wienerwaldensis</i>
ABOL_518_1	AMOL628-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_518_2	AMOL629-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_518_3	AMOL630-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_519_1	AMOL631-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_519_2	AMOL632-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_519_3	AMOL633-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_520_1	AMOL634-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_520_2	AMOL635-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_520_3	AMOL636-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_521	AMOL637-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_522_1	AMOL638-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_522_2	AMOL639-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_522_3	AMOL640-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_523_1	AMOL641-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_523_2	AMOL642-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_523_3	AMOL643-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_524_1	AMOL644-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_524_2	AMOL645-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_524_3	AMOL646-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_526_1	AMOL647-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_526_2	AMOL648-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_526_3	AMOL649-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_528_1	AMOL650-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_528_2	AMOL651-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_528_3	AMOL652-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_529_1	AMOL653-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_529_2	AMOL654-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_529_3	AMOL655-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_530_1	AMOL656-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_530_2	AMOL657-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_530_3	AMOL658-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_531_1	AMOL659-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_531_2	AMOL660-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_531_3	AMOL661-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_532_1	AMOL662-20	BOLD:AEC8473	<i>Hauffenia</i>	<i>kerschneri</i>
ABOL_534_1	AMOL663-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_534_2	AMOL664-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_534_3	AMOL665-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_536_1	AMOL666-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>

ID	BOLD numbers	BIN BOLD	Genus	Species
ABOL_539_1	AMOL667-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_543_1	AMOL668-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_543_2	AMOL669-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_543_3	AMOL670-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_544_1	AMOL671-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_544_2	AMOL672-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_544_3	AMOL673-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_546_1	AMOL674-20	BOLD:ADP3629	<i>Belgrandiella</i>	<i>aulaei</i>
ABOL_546_2	AMOL675-20	BOLD:ADP3629	<i>Belgrandiella</i>	<i>aulaei</i>
ABOL_546_3	AMOL676-20	BOLD:ADP3629	<i>Belgrandiella</i>	<i>aulaei</i>
ABOL_547_1	AMOL677-20	BOLD:AEC8473	<i>Hauffenia</i>	<i>kerschneri</i>
ABOL_560	AMOL678-20	BOLD:AAA4467	<i>Bythinella</i>	<i>conica</i>
ABOL_565_1	AMOL720-20	BOLD:AEC8473	<i>Hauffenia</i>	<i>kerschneri</i>
ABOL_566_1	AMOL721-20	BOLD:ADP3629	<i>Belgrandiella</i>	<i>aulaei</i>
ABOL_567_1	AMOL722-20	BOLD:ADP3629	<i>Belgrandiella</i>	<i>aulaei</i>
ABOL_567_2	AMOL723-20	BOLD:ADP3629	<i>Belgrandiella</i>	<i>aulaei</i>
ABOL_567_3	AMOL724-20	BOLD:ADP3629	<i>Belgrandiella</i>	<i>aulaei</i>



Supporting Figure 2: Neighbor joining tree with supporting bootstrap values (only values above 60 are displayed here) of all sequences, that are proposed by BOLD for the tree-based identification for the *Belgrandiella aulaei*, *Hauffenia wienerwaldensis* and *Hauffenia kerschneri* sequences. Blue: Sequences from specimen of the Kalkalpen National Park and its surroundings. Grey: BIN numbers

Supporting Table 7: Specimen overview of hydrobioid sequences from BOLD, that are used for the tree in Supporting Figure 2, including information about Process ID, Sample ID, BIN, identification, collecting date, country/ocean and region (if available).

Process ID	Sample ID	BIN	Identification	Collecting Date	Country/ Ocean	Region
GBMAB028-15	KT218519	BOLD:ACI1837	Belgrandiella krupensis			
GBMAB030-15	KT218518	BOLD:ACI1837	Belgrandiella krupensis			
GBMAB032-15	KT218515	BOLD:ACI1837	Belgrandiella krupensis			
GBMAB034-15	KT218517	BOLD:ACI1837	Belgrandiella krupensis			
GBMAB036-15	KT218516	BOLD:ACI1837	Belgrandiella krupensis			
GBMAB038-15	KT218520	BOLD:ACI1837	Belgrandiella kuseri			
AMOL048-18	ABOL-8_1	BOLD:ADP3629	Belgrandiella mimula	01.1.2009	Austria	Wiener Neustadt-Land
AMOL049-18	ABOL-8_2	BOLD:ADP3629	Belgrandiella mimula	01.1.2009	Austria	Wiener Neustadt-Land
AMOL568-18	AL-8056	BOLD:ADP3629	Belgrandiella sp.	27.10.2016	Austria	
AMOL674-20	ABOL-546_1	BOLD:ADP3629	Belgrandiella aulaei	17.10.2018	Austria	Steyr-Land
AMOL675-20	ABOL-546_2	BOLD:ADP3629	Belgrandiella aulaei	17.10.2018	Austria	Steyr-Land
AMOL676-20	ABOL-546_3	BOLD:ADP3629	Belgrandiella aulaei	17.10.2018	Austria	Steyr-Land
AMOL685-20	AL-8251	BOLD:ADP3629	Belgrandiella wawrai	13.6.2017	Austria	Baden
AMOL686-20	AL-8252	BOLD:ADP3629	Belgrandiella wawrai	13.6.2017	Austria	Baden
AMOL688-20	AL-8260	BOLD:ADP3629	Belgrandiella mimula	20.11.2019	Austria	Wiener Neustadt-Land
AMOL689-20	AL-8261	BOLD:ADP3629	Belgrandiella mimula	20.11.2019	Austria	Wiener Neustadt-Land
AMOL690-20	AL-8263	BOLD:ADP3629	Belgrandiella mimula	20.11.2019	Austria	Wiener Neustadt-Land
AMOL709-20	ABOL-56_1	BOLD:ADP3629	Belgrandiella wawrai	13.10.2012	Austria	Lilienfeld
AMOL710-20	ABOL-56_2	BOLD:ADP3629	Belgrandiella wawrai	13.10.2012	Austria	Lilienfeld
AMOL713-20	ABOL-105_1	BOLD:AED0666	Belgrandiella parreyssii	14.8.2014	Austria	Baden
AMOL714-20	ABOL-105_2	BOLD:AED0666	Belgrandiella parreyssii	14.8.2014	Austria	Baden
AMOL717-20	ABOL-389_1	BOLD:ADP3629	Belgrandiella fuchsi	24.7.2018	Austria	Lilienfeld
AMOL718-20	ABOL-389_2	BOLD:ADP3629	Belgrandiella fuchsi	24.7.2018	Austria	Lilienfeld
AMOL721-20	ABOL-566_1	BOLD:ADP3629	Belgrandiella aulaei	06.12.2019	Austria	Kirchdorf an der Krems
AMOL722-20	ABOL-567_1	BOLD:ADP3629	Belgrandiella aulaei	27.1.2020	Austria	Steyr-Land
AMOL723-20	ABOL-567_2	BOLD:ADP3629	Belgrandiella aulaei	27.1.2020	Austria	Steyr-Land
AMOL037-18	ABOL-52_1	BOLD:ADP3629	Belgrandiella wawrai	12.10.2012	Austria	Lilienfeld
GBMAB040-15	KT218514	BOLD:ACI1837	Belgrandiella zermanica			
GBMAB042-15	KT218513	BOLD:ACI1837	Belgrandiella zermanica			

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GBMAB044-15	KT218512	BOLD:ACI1837	Belgrandiella zermanica			
GBMAB046-15	KT218511	BOLD:ACI1837	Belgrandiella zermanica			
GBMAB048-15	KT218510	BOLD:ACI1837	Belgrandiella zermanica			
GBMAB708-15	KT218521	BOLD:ACI1837	Boleana umbilicata			
GBMLH1303-13	JX982796	BOLD:ACI1837	Boleana umbilicata		Slovenia	Mocilnik Spring
GBMLH1304-13	JX982795	BOLD:ACI1837	Boleana umbilicata		Slovenia	Mocilnik Spring
GBMLH0194-06	AF367641	BOLD:AAY2249	Graziana alpestris		Italy	Liguria, Savano, Molino, spring at the the Porra River
AMOL028-18	ABOL-37_2	BOLD:ADP3241	Graziana klagenfurtensis	01.1.2011	Austria	Klagenfurt Land
AMOL715-20	ABOL-388_1	BOLD:ADP3241	Graziana lacheineri	19.8.2018	Austria	Wolfsberg
AMOL716-20	ABOL-388_2	BOLD:ADP3241	Graziana lacheineri	19.8.2018	Austria	Wolfsberg
AMOL003-18	ABOL-100_1	BOLD:ADP4270	Graziana pupula	22.7.2014	Austria	Klagenfurt Land
AMOL053-18	ABOL-97_1	BOLD:ADP4270	Graziana pupula	16.7.2014	Austria	Klagenfurt-Land
AMOL194-18	AL-7466	BOLD:ADP4270	Graziana pupula	29.6.2015	Austria	
AMOL195-18	AL-7467	BOLD:ADP4270	Graziana pupula	29.5.2015	Austria	
AMOL221-18	AL-7523	BOLD:ADP2884	Graziana sp.	01.7.2015	Austria	
AMOL580-20	ABOL-484_1	BOLD:AEC8473	Hauffenia kerschneri	05.12.2018	Austria	Kirchdorf an der Krems
AMOL587-20	ABOL-503_1	BOLD:AEC8473	Hauffenia kerschneri	29.10.2018	Austria	Kirchdorf an der Krems
AMOL588-20	ABOL-503_2	BOLD:AEC8473	Hauffenia kerschneri	29.10.2018	Austria	Kirchdorf an der Krems
AMOL589-20	ABOL-503_3	BOLD:AEC8473	Hauffenia kerschneri	29.10.2018	Austria	Kirchdorf an der Krems
AMOL614-20	ABOL-512_1	BOLD:AEC8473	Hauffenia kerschneri	23.11.2018	Austria	Kirchdorf an der Krems
AMOL662-20	ABOL-532_1	BOLD:AEC8473	Hauffenia kerschneri	06.12.2018	Austria	Kirchdorf an der Krems
AMOL677-20	ABOL-547_1	BOLD:AEC8473	Hauffenia kerschneri	22.11.2018	Austria	Kirchdorf an der Krems
GBMLH1158-13	JF313940	BOLD:AAY2140	Hauffenia		Slovakia	Kunova Teplica, Slovensky Kras
GBML14708-19	KY087862	BOLD:ADR7660	Hauffenia erythropomatia		Slovenia	
GBML14709-19	KY087863	BOLD:ADR7659	Hauffenia erythropomatia		Slovenia	
GBML14710-19	KY087864	BOLD:ADR7658	Hauffenia media		Slovenia	
GBML14711-19	KY087865	BOLD:ADR7658	Hauffenia media		Slovenia	
GBMLH939-07	EF070614	BOLD:AAY2140	Hauffenia sp. Patrocnica spring		Slovakia	Patrocnica Spring, Gemerska Horka
GBML14712-19	KY087866	BOLD:ADR2331	Hauffenia subpiscinalis		Slovenia	
GBMAB1553-15	KT236156	BOLD:ACW1015	Hauffenia tellinii		Slovenia	Mocilnik Spring
GBML14713-19	KY087861	BOLD:ACW1015	Hauffenia tellinii		Slovenia	
GBMLH0193-06	AF367640	BOLD:AAY2141	Hauffenia tellinii		Italy	Friuli-Venetia-Julia
AMOL456-18	AL-8061	BOLD:ADP3094	Hauffenia wienerwaldensis	11.4.2017	Austria	

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AMOL625-20	ABOL-517_1	BOLD:ADP3094	<i>Hauffenia wienerwaldensis</i>	21.12.2018	Austria	Kirchdorf an der Krems
AMOL626-20	ABOL-517_2	BOLD:ADP3094	<i>Hauffenia wienerwaldensis</i>	21.12.2018	Austria	Kirchdorf an der Krems
AMOL627-20	ABOL-517_3	BOLD:ADP3094	<i>Hauffenia wienerwaldensis</i>	21.12.2018	Austria	Kirchdorf an der Krems
GBMLH1197-13	JF906765	BOLD:ACI2626	Hydrobiidae		Greece	Agrafa Mts
GBMLH1198-13	JF906764	BOLD:ACI2626	Hydrobiidae		Greece	Agrafa Mts
GBMLH1199-13	JF906763	BOLD:ACI2626	Hydrobiidae		Greece	Agrafa Mts
GBMLH1200-13	JF906762	BOLD:ACI2626	Hydrobiidae		Greece	Agrafa Mts
GBMLH1311-13	JX970610	BOLD:ACI1837	Hydrobiidae		Slovenia	Spring of Rakek
GBML14726-19	KY087875	BOLD:ADR6593	<i>Kerkia kareli</i>		Croatia	
GBML14727-19	KY087876	BOLD:ADR6593	<i>Kerkia kareli</i>		Croatia	
GBML14728-19	KY087877	BOLD:ADR6593	<i>Kerkia kareli</i>		Croatia	
GBML14730-19	KY087871	BOLD:ADR7660	<i>Kerkia</i> sp. Ljubac		Croatia	
GBML14732-19	KY087869	BOLD:ADR7660	<i>Kerkia</i> sp. Podgrade		Croatia	
GBML14733-19	KY087870	BOLD:ADR7660	<i>Kerkia</i> sp. Podgrade		Croatia	