Spring Ecosystems of the Alps: Isolated biodiversity islands with distinctive species assemblages

Inauguraldissertation

Zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Lucas Blattner

aus Basel, Schweiz

Basel, 2021

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

PD Dr. Stefanie von Fumetti

Departement Umweltwissenschaften
Geoökologie
Universität Basel

Prof. Dr. Moritz Lehmann

Departement Umweltwissenschaften
Aquatische und Isotopen-Biogeochemie
Universität Basel

PD Dr. Hans Jürgen Hahn

Institut für Umweltwissenschaften
Molekulare Ökologie
Universität Koblenz-Landau

Basel, den 19.10.2021

Prof. Dr. Marcel Mayor

(Dekan, Philosophisch-Naturwissenschaftliche Fakultät, Universität Basel)

Table of Contents

Summary

Freshwater springs are considered distinctive stream sections. Environmental conditions typical of subterranean groundwater habitats such as high temperature stability and nutrient scarcity predominate the spring mouth. Due to the rapidly increasing influence of surface processes such as solar radiation or precipitation downstream, springs exhibit steep environmental gradients and high microhabitat diversity on a small spatial scale. Furthermore, diverse species assemblages composed of crenobiontic (strictly spring-dwelling), stygobiontic (groundwater-adapted), and rhithrobiontic (stream-inhabiting) organisms are characteristic of springs. Due to their topographic distinctiveness and individual species assemblage compositions, particularly alpine springs are considered isolated island-like habitats and freshwater biodiversity hotspots.

Water mites (Hydrachnidia) show many crenobiontic species adapted to different microhabitats such as gravel, moss, lentic, or lotic flow regimes in springs and can be seen as a model system to study crenobiology. Their taxonomy is currently re-investigated intensively and is still mainly morphology-based, although molecular genetic methods can reveal novel insights. Therefore, I genetically assessed the a priori morphological species delimitation of the most abundant crenobiontic water mites in Chapter II. The phylogenetic inferences, including a mitochondrial (cox1) and a nuclear (28S) marker, corresponded to the traditional taxonomy and confirmed the monophyly of Hydrachnidia. Moreover, species putatively new to science were discovered, a genetic species identification reference database was generated, and basic methods for further genetic work were established.

In Chapter III, I then assessed the degree of spring habitat isolation by investigating the population genomic structure of *Partnunia steinmanni* Walter, 1906, a strictly crenobiontic water mite species. I sampled populations in protected areas across the Alps and performed restriction site-associated DNA sequencing (RADseq). The admixture analysis and an unrooted

phylogenetic tree revealed a pronounced population genomic structure and distinctiveness of *P. steinmanni* spring populations between and within the different areas. Combined with the strong isolation by distance that has been found, a high degree of insularity of alpine springs can be concluded. Furthermore, a genetic principal component analysis of individuals between the different protected areas revealed a western genotype extending into eastern populations, likely caused by post-glacial recolonization.

Finally, in Chapter IV, I developed and tested an environmental DNA (eDNA) bioindication methodology to monitor and assess alpine spring ecosystem integrity and detect a potential loss of crenobionts such as the species presented in Chapters II and III. For that purpose, I established novel qPCR primer and probe sets to detect indicator species in eDNA filtered water samples. The final assays targeting spring-dwelling Trichoptera, Plecoptera, and Hydrachnidia species showed to be highly specific and sensitive. Furthermore, equal detection rates were revealed by comparing the qPCR eDNA assays with the conventional approach, which relies on direct sampling and morphological identification of organisms. Due to its non-invasive and time-efficient character, the newly developed spring bioindication method circumvents drawbacks of the conventional techniques and is particularly applicable in protected areas.

Chapter I

General Introduction

A spring at Plan da l'Acqua Suot in the Swiss National Park

The Science of Springs and their Importance in the Anthropocene

Water is one of the most essential resources for human life, and springs play a substantial role as source of clean drinking water since the migration of hominins out of Africa and the evolution of modern humans (Cuthbert & Ashley, 2014). The earliest human settlements were predominantly built near accessible groundwater resources and freshwater springs that provided stable water supply and enabled the development of civilizations (Mithen, 2010; Stevanovic, 2010; Tegel et al., 2012; Knutsson, 2014). In modern urban areas, springs have been shown to provide suitable drinking water even during emergencies caused by water scarcity (Davis et al., 2020). Furthermore, their importance for remote rural communities, as well as the need for management strategies to preserve springs as an affordable water resource for cultures without access to advanced water purification technologies, has been strongly emphasized (Patton et al., 2020; Ranjan & Pandey, 2020).

Because of the high value of clean water for humankind, systematic investigations and documentations about groundwater and spring formation have been conducted early in human history. In the 1st century BC, the Roman Architect Marcus Vitruvius Pollio discussed in the 8th volume of his book series *De Architectura Libri Decem* how to locate springs and use them as a water resource (Stevanovic, 2010). In contrast to these more applied early records of springs and their appearance in nature, the French scientist and philosopher Pierre Perrault (17th century) was among the first researchers conducting empirical investigations on the origin of springs. In his book *De l' origine des fontaines* (1674) (Fig. 1), he concluded that springs originate from water, which reaches the

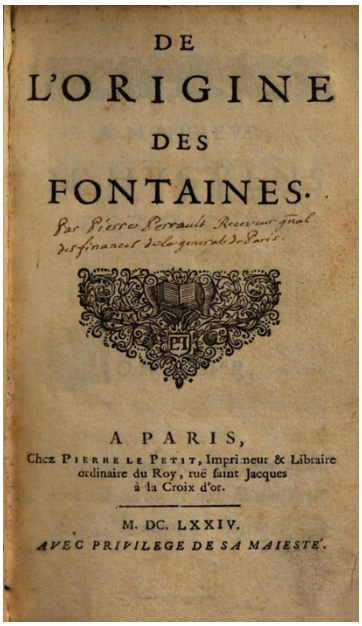

DE
L'ORIGINE
DES
FONTAINES.
Par P. Perrault Receveur gnal des finances & Intendant de Paris.
A PARIS,
Chez PIERRE LE PETIT, Imprimeur & Libraire ordinaire du Roy, ruë saint Jacques à la Croix d'or.
M. DC. LXXIV.
AVEC PRIVILEGE DE SA MAIESTÉ.

Fig. 1: Title page of P. Perrault's opus on the origin of springs written in 1674. One of the first scientific publications about empirical investigations on the geological and geomorphological processes behind spring formation.

ground in the form of precipitation and snowfall and is subsequently retained in soil layers (p. 148 in Perrault, 1674). To prove his assumption, Perrault conducted several experiments on the flow of water through soil and showed that soil is indeed capable of accumulating meteoric water (Perrault, 1674). His way of performing experiments to test his hypothesis and to empirically show that previous conclusions on the origin of springs have been premature pioneered the science of hydrology (Barontini & Settura, 2020).

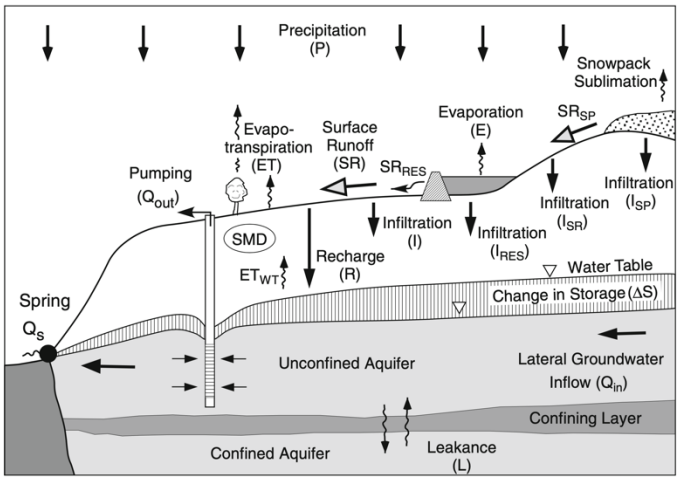


Fig. 2: Elements of the water budget in a spring drainage area.
I is infiltration in general, SR is surface water runoff, ET is evapotranspiration, I_{SR} is infiltration from surface runoff (including from sinking and losing streams), I_{RES} is infiltration from surface water reservoirs, I_{SP} is infiltration from snowpack and glaciers, R is groundwater recharge, SMD is soil moisture deficit, ET_{WT} is evapotranspiration from water table, Q_S is spring discharge Q_{IN} is lateral groundwater inflow to the aquifer feeding the spring, L is leakage back and forth between the underlying aquitard and the aquifer, Q_{OUT} is well pumpage from the aquifer, and S is change in storage of the aquifer.
(Kresic & Bonacci, 2010)

In contrast to the early periods of spring research, the anthropogenic influence on the water budget of aquifers has continuously increased and become a factor that needs to be taken into account when investigating the formation of springs today. Increased infiltration from surface water bodies due to precipitation or snowmelt leads to aquifer enrichment, whereas surface runoff and evapotranspiration diminish aquifer water availability and spring discharge (Kresic & Bonacci, 2010; Cochand et al., 2019) (Fig. 2). Depending on the biogeographic region and local topography, the processes governing groundwater formation, and thus spring flow intensity and permanence, undergo seasonal fluctuations and can differ even within small catchment areas as shown for montane and alpine drainage basins (Soulsby et al., 1999; Arnoux et al., 2020). In addition to these natural processes, groundwater overexploitation and mismanagement can further diminish aquifer water levels and impact the flow regime of springs. Intensive pumping combined with a reduced groundwater regeneration, e.g., due to sealed surfaces, decreased hydraulic conductivity due to colmation in agricultural areas, or increased evapotranspiration through land-use change, are

known to impact the water balance negatively and, therefore, diminish spring discharge (Brunke & Gonser, 1997; Seiler & Gat, 2007; Baillieux et al., 2015; Aslam et al., 2018; Bierkens & Wada, 2019; Levy et al., 2020; Faridatul & Bari, 2021). Human influence on springs, however, not only impacts their hydrology but consequently also affects the integrity of their ecosystem (Nielson et al., 2019).

Spring (crenal) habitats are defined as a distinct stream section followed by the rhithral (brooks and upper stream sections) and potamal (rivers and lower stream sections) (Illies & Botosaneanu, 1963). They harbour characteristic species assemblages (Cantonati et al., 2012b), which faunistically distinguish them from the other freshwater habitats (further elaborated in the following sections of the general introduction). Discharge is one of the main factors influencing spring fauna (Wood et al., 2005; Von Fumetti & Nagel, 2012), and some taxa such as spring-dwelling water mites completely disappear after desiccation events (Gerecke et al., 2018). As shown above, the hydrological consequences of human impact on the water table of aquifers mainly influence discharge and are therefore likely to impact the spring fauna and ecosystems. Furthermore, human-induced climate change is predicted to alter groundwater temperature (Aslam et al., 2018; Epting et al., 2021), which likely modifies the thermal regime of springs and downstream groundwater-dependent ecosystems. Aquatic organisms exhibit direct interactions with their environment, and physiological processes such as respiration are directly temperature-dependent (Verberk & Bilton, 2013; Ebner et al., 2019). Temperature-driven colonization patterns are likely in aquatic insects that exhibit physiological responses to changing thermal regimes and past climatic conditions (Ebner et al., 2020; McNamara et al., 2021). Therefore, spring-dwelling organisms are considered to be adapted to the relatively stable environmental conditions in springs (Cantonati et al., 2012b) and are likely to be influenced by climate change-induced environmental shifts. Overall, the interrelationships between natural and anthropogenic spring ecosystem alterations need to be taken into account when conducting crenobiological research and planning nature conservation strategies today.

Environmental and Geomorphological Characteristics of Springs

In its most elementary form, the definition of a spring habitat is reduced to the basic geomorphological phenomenon of subterranean groundwater that emerges from the aquifer and subsequently transforms into a surface water system (Van Der Kamp, 1995; Cantonati et al., 2012b; Glazier, 2014). The spring mouth (eucrenal) is dominated by environmental conditions characteristic for groundwater environments such as low-temperature variability and nutrient scarcity (Fattorini et al., 2016; Gerecke et al., 2018; Manenti & Piazza, 2021). Springs have thus been considered relatively stable environments and, therefore, even potential climate change refugia (Stubbington et al., 2009; Cartwright et al., 2020).

However, eucrenal temperature stability directly depends on the thermal regime of the aquifer and its depth, which can vary according to the local geological setting (Wood et al., 2005; Butscher & Huggenberger, 2007; Luhmann et al., 2011; Cantonati et al., 2020). Surface processes such as snow cover, precipitation, and air temperature can shape spring ecosystems and cause physicochemical conditions to change rapidly downstream, which results in steep environmental gradients on a small spatial scale (Kresic & Bonacci, 2010; Frisbee et al., 2013; Küry et al., 2016). Moreover, spring geomorphology and environmental conditions can be characteristic for specific biogeographic regions as shown for the major European ecoregions (Reiss et al., 2016).

Despite the generally high heterogeneity among different springs, various attempts have been made to classify spring habitats into distinctive types that share similar features as, among others, discharge, temperature, and geochemistry (for in-depth reviews see Glazier, 2014 and Stevens et al., 2021). Spring mouth geomorphology, at the transition point between subterranean and surface environment, is considered as a superordinate factor influencing spring ecosystems and therefore seen as surpassing spring classification criterion (Reiss et al., 2016; Stevens et al., 2021).

Steinmann et al. (1915) and Thienemann (1924) originally described three spring types that are widely used in crenobiology according to the geomorphological setting of groundwater emergence and topography (Cantonati et al., 2012b; Gerecke et al., 2018). Rheocrenes are lotic freshwater environments with gravity-driven groundwater emergence and a well-defined channel (Fig. 3, A). In contrast, diffuse flow and the formation of marsh-like surroundings are characteristic for lentic helocrenes (Fig. 3, C). Rheocrenes and helocrenes can both be formed on hillslopes or floodplains, respectively (Fig. 3, B). The third prominent appearance of springs, limnocrenes, are also lentic systems but with distinct pool formation through a gravity-driven focused flow (Fig. 3, D). Limnocrenic springs are mostly formed from geologic fissures or depressions (Meinzer, 1927; Odum, 1957). In addition to this well-defined basic classification system, intermediate spring types such as "rheohelocrenes" have been defined to account for the possibility of parallelly appearing lotic and lentic areas in a single spring (see e.g., Cantonati et al., 2006; Spitale et al., 2012). Furthermore, region-specific and geologically special spring types such as "hanging gardens" or geysers have been described (Stevens et al., 2021).

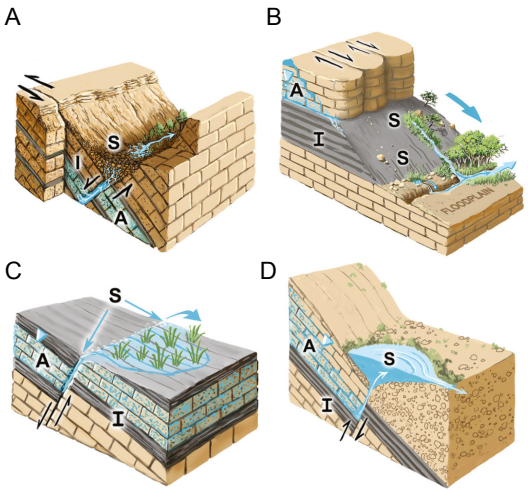


Fig. 3: Spring types.
Lotic Rheocrenes (A) and floodplain vs. hillslope (B). Lentic spring types: Helocrene (C) and limnocrene (D). On each figure, "A" stands for aquifer, "I" for impermeable infiltration barrier (aquitard), and "S" for surface groundwater expression (springs source). Modified from Stevens et al. (2021).

Springs as Biodiversity Hotspots

Freshwater spring ecosystems are not only rich in microhabitats and exhibit steep environmental gradients but also show exceptionally diverse species assemblages (Glazier, 2014; Cantonati et al., 2021). Strictly spring-dwelling species, so-called crenobionts, show relatively high diversity and are, among other taxa, numerous in Hydrachnidia (Gerecke et al.,

2018; Blattner et al., 2019; Pozojević et al., 2020), Diptera (Lencioni et al., 2011, 2012), Ostracoda (Rosati et al., 2017), Copepoda (Fattorini et al., 2016), diatoms (Cantonati et al., 2012a; Lai et al., 2020; Pascual et al., 2020), fungi (Wurzbacher et al., 2020) and Gastropoda (Falniowski et al., 2021).

Because crenic habitats appear at the interface between groundwater and surface water ecosystems, they are not exclusively inhabited by crenobiontic species. Stygophile (groundwater-related), and crenophile (spring-related) species (Fig. 4), as well as a hygrophilous fauna adapted to the interface between terrestrial and aquatic environments, are associated with springs (Cantonati et al., 2006).

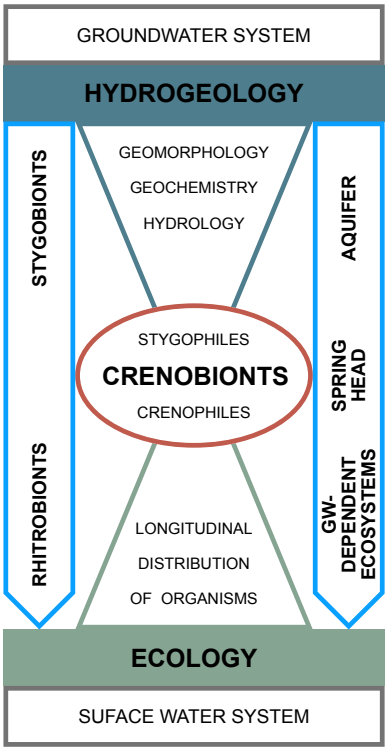


Fig. 4: Hourglass model of springs. The metaphoric hourglass model shows springs (red ellipsis) as transition ecosystems between groundwater (GW) and surface water environments. Species assemblages change from GW-restricted stygobionts, to crenobiontic, and rhithrobiontic (stream-dwelling) organisms that can be found in GW-dependent ecosystems. Modified from Cantonati et al. (2006, 2012b and 2020).

Due to the combined influence of subterranean and surface environments on springs (Fig. 4), a complex ensemble of biotic and abiotic factors can affect the spring biocenosis. Among others, flow permanence (Wood et al., 2005; Lai et al., 2020), discharge (Von Fumetti & Nagel, 2012), temperature (Barquín & Death, 2011; Lencioni et al., 2012; Von Fumetti et al., 2017), substrate availability (Hahn, 2000; Kubíková et al., 2012; Von Fumetti & Blattner, 2017), altitude (Wigger et al., 2015), food-web structure (Robinson et al., 2008), predation and competition (Di Sabatino et al., 2021), as well as nutrient availability (Lai et al., 2020) have been studied and discussed intensively and found to shape intra- and inter-spring species assemblages. Overall, springs are diverse ecosystems showing high biological niche availability and thus substantial species richness, respectively. Combined with their spatially restricted, often insular appearance (Cantonati et al., 2012b; Von Fumetti & Blattner, 2017; Cartwright et al., 2020), springs can overall be considered freshwater biodiversity hotspots.

Study System

Alpine Spring Ecosystems

Pioneer investigations were conducted in the 20th century by Nadig (1943), who investigated springs in the canton Grisons (Swiss Alps) and is assumed to be among the first authors describing alpine spring ecosystems (Reiss et al., 2016). The ecological setting and landscape history of alpine springs differ from other ecoregions (Cantonati et al., 2006; Gerecke et al., 2011; Reiss et al., 2016). Snowmelt seasonality and the permeability of Quaternary deposits like talus structures or moraines strongly shape alpine aquifers, resulting in springs with perennial (permanently flowing) or intermittent (periodically flowing) character (Arnoux et al., 2020). Temperature reflects this pattern and tends to be lower and more stable in perennial springs with a deeper aquifer, whereas intermittent and less stable springs can be found in shallow aquifers that are directly fed and influenced by snow melt (Küry et al., 2016; Arnoux et al., 2020). The altitude of springs further influences temperature, a general pattern of 0.3 – 0.5 °C decrease per 100 m has been reported (Wigger et al., 2015; Küry et al., 2016).

Species assemblages of alpine springs are composed of crenobionts as well as species that are characteristic for oligotrophic alpine headwater streams (Cantonati et al., 2006). Species richness and the abundance of crenobionts decrease with altitude, whereas cold-adapted rhithrobiontic species are predominant in springs at high altitudes (Wigger et al., 2015; Von Fumetti et al., 2017). This general pattern shows that crenobionts may be less susceptible to the expected climate change-induced temperature rise in the Alps (Gobiet et al., 2020) than the cold-adapted alpine rhithrobionts (Von Fumetti et al., 2017). This assumption is further supported by the results of a study investigating proteome response to increased temperature in the crenobiontic caddisfly *Crunoecia irrorata* (Curtis, 1834), which concluded overall temperature resilience (Ebner et al., 2019).

The Alps exhibited multiple ice-age periods and have likely been completely covered by Pleistocene glaciers during the last glacial maximum (LGM) (Kelly et al., 2004; Seguinot et al., 2018). Consequently, most freshwater species disappeared, and after the retreat of the glaciers during the Holocene, approximately 11'700 years BP, have experienced a history of re-colonisation predominantly from eastern and western ice-free refugia (Hewitt, 2000; Walker et al., 2009; Reiss et al., 2016). Despite these general patterns, it is assumed that aquatic organisms have diverse re-colonisation routes and are likely to show multiple in situ refugia, which caused high genetic isolation and endemism (Malicky, 1983; Pauls et al., 2006). Therefore, habitat insularity and resulting high genetic diversity are particularly assumed in springs of the Alps (Cantonati et al., 2006; Fattorini et al., 2016; Von Fumetti & Blattner, 2017). This highlights their importance as valuable freshwater ecosystems and the need for extended conservation efforts as well as monitoring campaigns to track biotic and abiotic change over time (Robinson & Oertli, 2009; Gerecke et al., 2011; Cartwright, 2019; Lechner et al., 2020; Cantonati et al., 2021).

Water Mites (Hydrachnidia)

As shown in the above-section *Springs as Biodiversity Hotspots,* the crenocenosis is composed of different taxa including, among others, Insecta, Chelicerata, Crustacea, and Turbellaria (e.g., Cantonati et al., 2020 and references therein). Water mites (Chelicerata, Acari, Trombidiformes: Hydrachnidia) are considered among the most diverse organisms inhabiting crenic habitats and show many species strictly bound to springs while being completely absent in downstream sections (Gerecke et al., 2018). Among the approximately 970 water mite species that have been recorded in Europe until now, 137 species can be found solely in springs, adapted to different microhabitats such as moss, gravel, sand, lotic, and lentic environments (Di Sabatino et al., 2003; Gerecke et al., 2018). Water mite taxonomy is intensively re-evaluated

today and genetic species delimitations methods, which increasingly start playing a role in water mite taxonomy, reveal an underestimation of species diversity and the potential of many species new to science (Martin et al., 2010; Pešić et al., 2017; Blattner et al., 2019; Alarcon-Elbal et al., 2020; Montes-Ortiz & Elías-Gutiérrez, 2020). Water mites show complex interactions with spring ecosystems, mainly due to their parasitic live stage (Gerecke & Di Sabatino, 2007; Martin et al., 2009). After hatching, mite larvae of nearly all species obligatorily parasitize aquatic insect hosts, feed on their body fluids, and disperse by using the host's ability to fly (Andrés & Cordero, 1998; Di Sabatino et al., 2000; Gerecke et al., 2020) (Fig. 5). Subsequently, after the parasitic stage, larvae return to the water and undergo three nymphal stages composed of an inactive protonymph, a predatory deutonymph and a last inactive tritonymph live-stage before becoming adult (Martin & Gerecke, 2009) (Fig. 5). Because many species are adapted to various microhabitats in springs (Di Sabatino et al., 2000; Fernández & Fossati, 2011), and exhibit complex interrelationships with other aquatic organisms, Hydrachnidia are considered ideal bioindicators and a model system to study crenobiology (Więcek et al., 2013; Goldschmidt, 2016; Zawal et al., 2018; Pozojević et al., 2020).

Fig. 5: Hydrachnidia life-cycle.
(Martin & Gerecke, 2009)

Scientific method development in water mite research is, however, still in its infancy. Genetic species delimitation, for example, is dominated by traditional markers (e.g., Dabert et al., 2016; Stryjecki et al., 2016). High throughput sequencing technologies have been focused on prey-species range reconstruction (Martin et al., 2015; Vasquez et al., 2021), and NCBI (accessed 09.2021) genomic data is restricted to few mitochondrial genomes with no fully assembled and annotated complete genome available. The diverse "omics" approaches thus still offer new possibilities to advance water mite research in the future and study their interaction with spring habitats.

Aim and Outline

The proposed ecological value of spring ecosystems has not only been based on the overall high diversity of spring-dwelling species, their habitat specificity, and therefore power as bioindicators for changing environmental conditions. A high degree of spring habitat isolation and insularity, especially in alpine environments (Von Fumetti & Blattner, 2017 and references therein), is considered as an additional major characteristic of springs. However, studies investigating the interconnection of alpine crenic habitats are scarce and empirical data focus on broader colonization patterns, or are available only for species that are not strictly spring-dwelling (e.g., Ward, 1994; Pauls et al., 2006; Benke et al., 2011; Engelhardt et al., 2011).

Among the different taxa that show species present in springs, water mites are of particular interest to study the degree of isolation between springs because of their high degree of habitat restrictedness and rather weak dispersal abilities (Di Sabatino et al., 2003; Gerecke et al., 2018). Species description, however, has been mainly morphology-based (e.g., Stryjecki et al., 2016), and molecular genetic methods to investigate habitat interconnection need prior evaluation. To gain insight into possible discrepancies between a priori morphospecies vs. genetic species assignments and develop a base of knowledge for genomic data generation, I investigated genetic distances and the phylogenetic placement of a broad set of representative crenobiontic water mite species in **Chapter II**. On this basis, first genetic diversity patterns were revealed. Furthermore, a genetic species delimitation database for spring water mite species was established to be able to choose a model species suitable to research the degree of alpine spring habitat interconnection and isolation.

I then evaluated this fundamental assumption in crenobiology in **Chapter III**. For this purpose, I sampled *Partnunia steinmanni* Walter, 1906 populations on a relatively broad geographic scale in different protected areas across the Alps. Subsequently, I generated a high-

throughput sequencing data set and inferred population genomic structure to gain novel insight into the admixture, phylogenomic clade association and genetic differentiation as well as isolation by distance among the sampled spring populations.

Finally, I developed a new non-invasive tool to detect the presence of bioindicator species in environmental DNA (eDNA) filtered water samples in **Chapter IV**, providing a state-of-the-art methodology that can be used to assess and monitor alpine spring ecosystems. I showed for the first time that eDNA bioindication methods are fully applicable in springs and which methodological challenges need to be taken into account compared to other environments. This newly developed bioindication tool supplements conventional approaches, circumvents their major drawbacks and is of particular interest for protected areas because of its low invasiveness.

Overall, **Chapter II** gives insight into spring-dwelling water mite species diversity, taxonomy, and phylogeny, re-evaluates species assignments, and provides the basic methods to conduct further genomic work. **Chapter III** contributes to the understanding of intra-specific genetic distinctiveness of crenobiontic species and provides first direct empirical data on the degree of spring habitat isolation on an alpine scale. A novel method to monitor and assess spring ecosystems and detect a possible loss of the species presented in Chapter II and III, as well as other organisms that are indicative of spring ecosystem integrity, is presented in **Chapter IV.**

References

Alarcon-Elbal, P., R. Garcia-Jimenez, M. Pelaez, J. L. Horreo, & A. G. Valdecasas, 2020. Molecular Correlation between Larval , Deutonymph and Adult Stages of the Water Mite Arrenurus (Micruracarus) Novus. *MDPI Live* , https://doi.org/10.3390/life10070108.

Andrés, J. A., & A. Cordero, 1998. Effects of water mites on the damselfly Ceriagrion tenellum. *Ecological Entomology* 23: 103–109, https://doi.org/10.1046/j.1365-2311.1998.00125.x.

Arnoux, M., L. J. S. Halloran, E. Berdat, & D. Hunkeler, 2020. Characterizing seasonal groundwater storage in alpine catchments using time-lapse gravimetry, water stable isotopes and water balance methods. *Hydrological Processes* 34: 4319–4333, https://doi.org/10.1002/hyp.13884.

Aslam, R. A., S. Shrestha, & V. P. Pandey, 2018. Groundwater vulnerability to climate change: A review of the assessment methodology. *Science of the Total Environment* Elsevier B.V. 612: 853–875, https://doi.org/10.1016/j.scitotenv.2017.08.237.

Baillieux, A., C. Moeck, P. Perrochet, & D. Hunkeler, 2015. Estimer l'évolution de la qualité d'une eau souterraine par fonctions de transfert. *Hydrogeology Journal* 23: 1449–1463, https://doi.org/10.1007/s10040-015-1279-5.

Barontini, S., & M. Settura, 2020. Beyond Perrault's experiments: Repeatability, didactics and complexity. *Hydrology and Earth System Sciences* 24: 1907–1926, https://doi.org/10.5194/hess-24-1907-2020.

Barquín, J., & R. G. Death, 2011. Downstream changes in spring-fed stream invertebrate communities: The effect of increased temperature range?. *Journal of Limnology* 70: 134–146, https://doi.org/10.3274/JL11-70-S1-10.

Benke, M., M. Brändle, C. Albrecht, & T. Wilke, 2011. Patterns of freshwater biodiversity in Europe: Lessons from the spring snail genus Bythinella. *Journal of Biogeography* 38: 2021–2032, https://doi.org/10.1111/j.1365-2699.2011.02527.x.

Bierkens, M. F. P., & Y. Wada, 2019. Non-renewable groundwater use and groundwater depletion: A review. *Environmental Research Letters* IOP Pub., https://doi.org/10.1088/1748-9326/ab1a5f.

Blattner, L., R. Gerecke, & S. Von Fumetti, 2019. Hidden biodiversity revealed by integrated morphology and genetic species delimitation of spring dwelling water mite species (Acari, Parasitengona: Hydrachnidia). *Parasites and Vectors* BioMed Central 12: 1–13, https://doi.org/10.1186/s13071-019-3750-y.

Brunke, M., & T. Gonser, 1997. The ecological significance of exchange processes between rivers and groundwater. *Freshwater Biology* 37: 1–33, https://doi.org/10.1046/j.1365-2427.1997.00143.x.

Butscher, C., & P. Huggenberger, 2007. Implications for karst hydrology from 3D geological modeling using the aquifer base gradient approach. *Journal of Hydrology* 342: 184–198, https://doi.org/10.1016/j.jhydrol.2007.05.025.

Cantonati, M., N. Angeli, E. Bertuzzi, D. Spitale, & H. Lange-Bertalot, 2012a. Diatoms in springs of the Alps: Spring types, environmental determinants, and substratum. *Freshwater Science* 31: 499–524, https://doi.org/10.1899/11-065.1.

Cantonati, M., R. J. Fensham, L. E. Stevens, R. Gerecke, D. S. Glazier, N. Goldscheider, R. L. Knight, J. S. Richardson, A. E. Springer, & K. Tockner, 2021. Urgent plea for global protection of springs. *Conservation Biology* 35: 378–382, https://doi.org/10.1111/cobi.13576.

Cantonati, M., L. Füreder, R. Gerecke, I. Jüttner, & E. J. Cox, 2012b. Crenic habitats, hotspots for freshwater biodiversity conservation: toward an understanding of their ecology. *Freshwater Science* 31: 463–480, https://doi.org/10.1899/11-111.1.

Cantonati, M., R. Gerecke, & E. Bertuzzi, 2006. Springs of the Alps – Sensitive Ecosystems to Environmental Change: From Biodiversity Assessments to Long-term Studies. *Hydrobiologia* 562: 59–96, https://doi.org/10.1007/s10750-005-1806-9.

Cantonati, M., L. E. Stevens, S. Segadelli, A. E. Springer, N. Goldscheider, F. Celico, M. Filippini, K. Ogata, & A. Gargini, 2020. Ecohydrogeology: The interdisciplinary convergence needed to improve the study and stewardship of springs and other groundwater-dependent habitats, biota, and ecosystems. *Ecological Indicators* Elsevier 110: 105803, https://doi.org/10.1016/j.ecolind.2019.105803.

Cartwright, J., 2019. Ecological islands: conserving biodiversity hotspots in a changing climate. *Frontiers in Ecology and the Environment* 17: 331–340, https://doi.org/10.1002/fee.2058.

Cartwright, J., K. Dwire, Z. Freed, S. Hammer, B. McLaughlin, L. Misztal, E. Schenk, J. Spence, A. Springer, & L. Stevens, 2020. Oases of the future? Springs as potential hydrologic refugia in drying climates. *Frontiers in Ecology and the Environment* 18: 245–253, https://doi.org/10.1002/fee.2191.

Cochand, M., P. Christe, P. Ornstein, & D. Hunkeler, 2019. Groundwater Storage in High Alpine Catchments and Its Contribution to Streamflow. *Water Resources Research* 55: 2613–2630, https://doi.org/10.1029/2018WR022989.

Cuthbert, M. O., & G. M. Ashley, 2014. A spring forward for hominin evolution in East Africa. *PLoS ONE* 9:, https://doi.org/10.1371/journal.pone.0107358.

Dabert, M., H. Proctor, & J. Dabert, 2016. Higher-level molecular phylogeny of the water mites (Acariformes: Prostigmata: Parasitengonina: Hydrachnidiae). *Molecular Phylogenetics and Evolution* Elsevier Inc. 101: 75–90, https://doi.org/10.1016/j.ympev.2016.05.004.

Davis, D., D. Diadin, A. Shores, O. Khandogina, & M. Laituri, 2020. Capacity of urban springs to support emergency water needs, a secondary city case study: Kharkiv, Ukraine. *Urban Water Journal* Taylor & Francis 17: 368–376, https://doi.org/10.1080/1573062X.2020.1764064.

Di Sabatino, A., B. Cicolani, & R. Gerecke, 2003. Biodiversity and distribution of water mites (Acari, Hydrachnidia) in spring habitats. *Freshwater Biology* 48: 2163–2173, https://doi.org/10.1046/j.1365-2427.2003.01151.x.

Di Sabatino, A., L. Coscieme, F. P. Miccoli, & G. Cristiano, 2021. Benthic invertebrate assemblages and leaf-litter breakdown along the eucrenal–hypocrenal ecotone of a rheocrene spring in Central Italy: Are there spatial and seasonal differences?. *Ecohydrology* 1–11, https://doi.org/10.1002/eco.2289.

Di Sabatino, A., R. Gerecke, & P. Martin, 2000. The biology and ecology of lotic water mites (Hydrachnidia). *Freshwater Biology* 44: 47–62.

Ebner, J. N., D. Ritz, & S. Von Fumetti, 2019. Comparative proteomics of stenotopic caddisfly Crunoecia irrorata identifies acclimation strategies to warming. *Molecular Ecology* , https://doi.org/10.1111/mec.15225.

Ebner, J. N., D. Ritz, & S. von Fumetti, 2020. Abiotic and past climatic conditions drive protein abundance variation among natural populations of the caddisfly Crunoecia irrorata. *Scientific Reports* Nature Publishing Group UK 10: 1–15, https://doi.org/10.1038/s41598-020-72569-4.

Engelhardt, C. H. M., P. Haase, & S. U. Pauls, 2011. From the Western Alps across Central Europe: Postglacial recolonisation of the tufa stream specialist Rhyacophila pubescens (Insecta, Trichoptera). *Frontiers in Zoology* 8: 1–14, https://doi.org/10.1186/1742-9994-8-10.

Epting, J., A. Michel, A. Affolter, & P. Huggenberger, 2021. Climate change effects on groundwater recharge and temperatures in Swiss alluvial aquifers. *Journal of Hydrology X* Elsevier B.V. 11: 100071, https://doi.org/10.1016/j.hydroa.2020.100071.

Falniowski, A., B. Lewarne, A. Rysiewska, A. Osikowski, & S. Hofman, 2021. Crenobiont, stygophile and stygobiont molluscs in the hydrographic area of the trebišnjica river basin. *ZooKeys* 2021: 61–89, https://doi.org/10.3897/zookeys.1047.64034.

Faridatul, M. I., & M. N. Bari, 2021. Understanding the Long-Term Changes in Groundwater Level—A Tale of Highly Urbanized City. *Journal of Geographic Information System* 13: 466–484, https://doi.org/10.4236/jgis.2021.134026.

Fattorini, S., P. A. V. Borges, B. Fiasca, & D. M. P. Galassi, 2016. Trapped in the web of water: Groundwater-fed springs are island-like ecosystems for the meiofauna. *Ecology and Evolution* 6: 8389–8401, https://doi.org/10.1002/ece3.2535.

Fernández, H. R., & O. Fossati, 2011. An initial classification of neotropical water mites (Acari: Hydrachnidia) based on habitat preferences. *International Journal of Ecology* 2011: 1–12, https://doi.org/10.1155/2011/910540.

Frisbee, M. D., F. M. Phillips, A. F. White, A. R. Campbell, & F. Liu, 2013. Effect of source integration on the geochemical fluxes from springs. *Applied geochemistry* Elsevier 28: 32–54.

Gerecke, R., M. Cantonati, D. Spitale, E. Stur, & S. Wiedenbrug, 2011. The challenges of long-term ecological research in springs in the northern and southern Alps: Indicator groups, habitat diversity, and medium-term change. *Journal of Limnology* 70: 168–187, https://doi.org/10.3274/JL11-70-S1-13.

Gerecke, R., & A. Di Sabatino, 2007. Water mites (Hydrachnidia and Halacaridae) in Spring habitats: a taxonomical and ecological perspective In Cantonati, M., E. Bertruzzi, & D. Spitale (eds), The spring habitat: Biota and sampling methods. Museo Tridentino di Scienze Naturali, Trento: 193–216, https://doi.org/10.1002/qua.10723.

Gerecke, R., P. Martin, & T. Gledhill, 2018. Water mites (Acari Parasitengona: Hydrachnidia) as inhabitants of groundwater-influenced habitats - considerations following an update of Limnofauna Europaea. *Limnologica* , https://doi.org/10.1016/j.limno.2017.11.008.

Gerecke, R., A. Wohltmann, B. P. Smith, & M. Judson, 2020. New taxa of the water mite family Limnocharidae (Actinotrichida : Eylaoidea) parasitising tropical water bugs of the genus Rhagovelia Mayr, 1865 (Hemiptera : Veliidae) reveal unsuspected diversity of larval morphologies. *Aquatic Insects* 1865:, https://doi.org/10.1080/01650424.2020.1811876.

Glazier, D. S., 2014. Springs. Reference Module in Earth Systems and Environmental Sciences. Elsevier, https://doi.org/10.1016/B978-0-12-409548-9.09322-2.

Gobiet, A., S. Kotlarski, A. Gobiet, & S. Kotlarski, 2020. Future Climate Change in the European Alps. *Oxford Research Encyclopedia of Climate Science* 1–23, https://doi.org/https://doi.org/10.1038/s41558-019-0676-5.

Goldschmidt, T., 2016. Water mites (Acari, Hydrachnidia): powerful but widely neglected bioindicators – a review. *Neotropical Biodiversity* Taylor & Francis 2: 12–25, https://doi.org/10.1080/23766808.2016.1144359.

Hahn, H. J., 2000. Studies on classifying of undisturbed spring in Southwestern Germany by macrobenthic communities. *Limnologica - Ecology and Management of Inland Waters* 30: 247–259, https://doi.org/10.1016/S0075-9511(00)80055-9.

Hewitt, G., 2000. The genetic legacy of the quaternary ice ages. *Nature* 405: 907–913, https://doi.org/10.1038/35016000.

Illies, J., & L. Botosaneanu, 1963. Problèmes et méthodes de la classification et de la zonation écologique des eaux courantes, considérées surtout du point de vue faunistique. *Mitteilungen der Internationalen Vereinigung für theoretische und angewandte Limnologie* 12: 1–57.

Kelly, M. A., J. F. Buoncristiani, & C. Schlüchter, 2004. A reconstruction of the last glacial maximum (LGM) ice-surface geometry in the western Swiss Alps and contiguous Alpine regions in Italy and France. *Eclogae Geologicae Helvetiae* 97: 57–75, https://doi.org/10.1007/s00015-004-1109-6.

Knutsson, G., 2014. The role of springs in the history of Sweden. *Journal of Water Management and Research* 70: 79–86.

Kresic, N., & O. Bonacci, 2010. Spring discharge hydrograph In Kresic, N., & Z. Stevanovic (eds), Groundwater Hydrology of Springs. : 129–162.

Kubíková, L., O. P. Simon, K. Tichá, K. Douda, M. Maciak, & M. Bílý, 2012. The influence of mesoscale habitat conditions on the macroinvertebrate composition of springs in a geologically homogeneous area. *Freshwater Science* 31: 668–679, https://doi.org/10.1899/11-174.1.

Küry, D., V. Lubini, & P. Stucki, 2016. Temperature patterns and factors governing thermal response in high elevation springs of the Swiss Central Alps. *Hydrobiologia* , https://doi.org/10.1007/s10750-016-2918-0.

Lai, G. G., B. M. Padedda, L. Ector, C. E. Wetzel, A. Lugliè, & M. Cantonati, 2020. Mediterranean karst springs: Diatom biodiversity hotspots under the pressure of hydrological fluctuation and nutrient enrichment. *Plant Biosystems* Taylor & Francis 154: 673–684, https://doi.org/10.1080/11263504.2019.1674402.

Lechner, K., M. Ribis, & G. Poscher, 2020. High-discharge springs of the Karwendel massif (Northern Limestone Alps, Tyrol): Contribution to the understanding of hydrogeological processes to ensure the future drinking-water supply. *Grundwasser - Zeitschrift der Fachsektion Hydrogeologie* 25: 71–80, https://doi.org/10.1007/s00767-019-00440-z.

Lencioni, V., L. Marziali, & B. Rossaro, 2011. Diversity and distribution of chironomids (Diptera, Chironomidae) in pristine Alpine and pre-Alpine springs (Northern Italy). *Journal of Limnology* 70: 106–121, https://doi.org/10.3274/JL11-70-S1-08.

Lencioni, V., L. Marziali, & B. Rossaro, 2012. Chironomids as bioindicators of environmental quality in mountain springs. *Freshwater Science* 31: 525–541, https://doi.org/10.1899/11-038.1.

Levy, Y., A. Burg, Y. Yechieli, & H. Gvirtzman, 2020. Displacement of springs and changes in groundwater flow regime due to the extreme drop in adjacent lake levels: The Dead Sea rift. *Journal of Hydrology* Elsevier 587: 124928, https://doi.org/10.1016/j.jhydrol.2020.124928.

Luhmann, A. J., M. D. Covington, A. J. Peters, S. C. Alexander, C. T. Anger, J. A. Green, A. C. Runkel, & E. C. Alexander, 2011. Classification of thermal patterns at karst springs and cave streams. *Ground Water* Wiley Online Library 49: 324–335.

Malicky, H., 1983. Chorological patterns and biome types of European Trichoptera and other freshwater insects. *Archiv für Hydrobiologie* 96: 223–244.

Manenti, R., & B. Piazza, 2021. Between darkness and light : Spring habitats provide new perspectives for modern researchers on groundwater biology. *PeerJ* 1–18, https://doi.org/10.7717/peerj.11711.

Martin, P., M. Dabert, & J. Dabert, 2010. Molecular evidence for species separation in the water mite Hygrobates nigromaculatus Lebert, 1879 (Acari, Hydrachnidia): Evolutionary consequences of the loss of larval parasitism. *Aquatic Sciences* 72: 347–360, https://doi.org/10.1007/s00027-010-0135-x.

Martin, P., & R. Gerecke, 2009. Diptera as hosts of water mite larvae - an interesting relationship with many open questions tionship with many open questions. *Lauterbornia* 68: 95–103.

Martin, P., M. Koester, L. Schynawa, & R. Gergs, 2015. First detection of prey DNA in Hygrobates fluviatilis (Hydrachnidia, Acari): A new approach for determining predator–prey relationships in water mites. *Exp. and Applied Acarology* 67:373–380, https://doi.org/10.1007/s10493-015-9956-6.

Martin, P., E. Stur, & S. Wiedenbrug, 2009. Larval parasitism of spring-dwelling alpine water mites (Hydrachnidia, Acari): A study with particular reference to chironomid hosts. *Aquatic Ecology* 44: 431–448, https://doi.org/10.1007/s10452-009-9301-4.

McNamara, S. C., M. R. Pintar, & W. J. Resetarits, 2021. Temperature but not nutrient addition affects abundance and assemblage structure of colonizing aquatic insects. *Ecology* 102: 1–12, https://doi.org/10.1002/ecy.3209.

Meinzer, O. E., 1927. Large springs in the United States. Washington, https://doi.org/https://doi.org/10.3133/wsp557.

Mithen, S., 2010. The domestication of water: Water management in the ancient world and its prehistoric origins in the Jordan Valley. *Philosophical Transactions of the Royal Society* 368: 5249–5274, https://doi.org/10.1098/rsta.2010.0191.

Montes-Ortiz, L., & M. Elías-Gutiérrez, 2020. Water Mite Diversity (Acariformes: Prostigmata: Parasitengonina: Hydrachnidiae) from Karst Ecosystems in Southern of Mexico: A Barcoding Approach. *Diversity* 12: 329, https://doi.org/10.3390/d12090329.

Nadig, A., 1943. Hydrobiologische Untersuchungen in Quellen des Schweizerischen Nationalparks im Engadin. *Ergebnisse der wissenschaftlichen Untersuchungen im Schweizer Nationalpark* 1: 67–432.

Nielson, K. G., K. M. Gill, A. E. Springer, J. D. Ledbetter, L. E. Stevens, & S. B. Rood, 2019. Springs ecosystems: vulnerable ecological islands where environmental conditions, life history traits, and human disturbance facilitate non-native plant invasions. *Biological Invasions* Springer International Publishing 21: 2963–2981, https://doi.org/10.1007/s10530-019-02025-6.

Odum, H. T., 1957. Trophic structure and productivity of Silver Springs, Florida. *Ecological monographs* Wiley Online Library 27: 55–112.

Pascual, R., A. Nebra, J. Gomà, C. Pedrocchi, & O. Cadiach, 2020. First data on the biological richness of Mediterranean springs. *Limnetica* 39: 121–139, https://doi.org/10.23818/limn.39.09.

Patton, H., L. A. Krometis, & E. Sarver, 2020. Springing for safe water: Drinking water quality and source selection in central appalachian communities. *Water (Switzerland)* 12:, https://doi.org/10.3390/w12030888.

Pauls, S. U., H. T. Lumbsch, & P. Haase, 2006. Phylogeography of the montane caddisfly Drusus discolor: Evidence for multiple refugia and periglacial survival. *Molecular Ecology* 15: 2153–2169, https://doi.org/10.1111/j.1365-294X.2006.02916.x.

Perrault, P., 1674. De l'origine des fontaines. Chez Pierre le Petit, imprimeur & libraire ordinaire du Roy, ruë Sain Jaques à la Croix s' or, Paris.

Pešić, V., M. Asadi, M. Cimpean, M. Dabert, Y. Esen, R. Gerecke, P. Martin, A. Savić, H. Smit, & E. Stur, 2017. Six species in one: Evidence of cryptic speciation in the Hygrobates fluviatilis complex (Acariformes, Hydrachnidia, Hygrobatidae). *Systematic and Applied Acarology* 22: 1327–1337, https://doi.org/10.11158/saa.22.9.4.

Pozojević, I., V. Pešić, T. Goldschmidt, & S. Gottstein, 2020. Crenal habitats: Sources of water mite (Acari: Hydrachnidia) diversity. *Diversity* 12: 19–23, https://doi.org/10.3390/D12090316.

Ranjan, P., & K. P. Pandey, 2020. Reviving, Development and Protection of Springs to Increase Water Security in the Himalayan Region. *SSRN Electronic Journal* 25–29, https://doi.org/10.2139/ssrn.3516630.

Reiss, M., P. Martin, R. Gerecke, & S. von Fumetti, 2016. Limno-ecological characteristics and distribution patterns of spring habitats and invertebrates from the Lowlands to the Alps. *Environmental Earth Sciences* Springer Berlin Heidelberg 75: 1–11, https://doi.org/10.1007/s12665-016-5818-8.

Robinson, C. T., & B. Oertli, 2009. Long-term biomonitoring of alpine waters in the Swiss National Park. *Eco.mont* 1: 23–34, https://doi.org/10.1553/eco.mont1s23.

Robinson, C. T., D. Schmid, M. Svoboda, & S. M. Bernasconi, 2008. Functional measures and food webs of high elevation springs in the Swiss alps. *Aquatic Sciences* 70: 432–445, https://doi.org/10.1007/s00027-008-8125-y.

Rosati, M., G. Rossetti, M. Cantonati, V. Pieri, J. R. Roca, & F. Mesquita-Joanes, 2017. Are aquatic assemblages from small water bodies more stochastic in dryer climates? An analysis of ostracod spring metacommunities. *Hydrobiologia* Springer 793: 199–212.

Seguinot, J., S. Ivy-Ochs, G. Jouvet, M. Huss, M. Funk, & F. Preusser, 2018. Modelling last glacial cycle ice dynamics in the Alps. *Cryosphere* 12: 3265–3285, https://doi.org/10.5194/tc-12-3265-2018.

Seiler, K.-P., & J. R. Gat, 2007. Groundwater Recharge from Run-off, Infiltration and Percolation. Springer Netherlands, https://doi.org/10.1007/978-1-4020-5306-1.

Soulsby, C., R. Malcolm, R. Helliwell, & R. Ferrier, 1999. Hydrogeochemistry of montane springs and their influence on streams in the Cairngorm mountains, Scotland. *Hydr. and Earth System Sci.*: https://doi.org/https://doi.org/10.5194/hess-3-409-1999.

Spitale, D., M. Leira, N. Angeli, & M. Cantonati, 2012. Environmental classification of springs of the Italian Alps and its consistency across multiple taxonomic groups. *Freshwater Science* 31: 563–574, https://doi.org/10.1899/10-038.1.

Steinmann, P., R. Siegrist, & H. Gams, 1915. Praktikum der Süßwasserbiologie. Gebr. Borntraeger.

Stevanovic, Z., 2010. Utilization and regulation of springs In Kresic, N., & Z. Stevanovic (eds), Groundwater Hydrology of Springs. Elsevier, https://doi.org/https://doi.org/10.1016/C2009-0-19145-6.

Stevens, L. E., E. R. Schenk, & A. E. Springer, 2021. Springs ecosystem classification. *Ecological Applications* 31:, https://doi.org/10.1002/eap.2218.

Stryjecki, R., A. Bańkowska, M. Gryzińska, E. Sarnacka, M. Rutkowska, & A. Zawal, 2016. The use of molecular techniques in the taxonomy of water mites (Hydrachnidia, Acari). *Acta Biologica* 23: 23–10, https://doi.org/10.18276/ab.2016.23-10.

Stubbington, R., A. M. Greenwood, P. J. Wood, P. D. Armitage, J. Gunn, & A. L. Robertson, 2009. The response of perennial and temporary headwater stream invertebrate communities to hydrological extremes. *Hydrobiologia* 630: 299–312, https://doi.org/10.1007/s10750-009-9823-8.

Tegel, W., R. Elburg, D. Hakelberg, H. Stäuble, U. Büntgen, & M. D. Petraglia, 2012. Early Neolithic Water Wells Reveal the World's Oldest Wood Architecture. *PLoS ONE* 7: 1–8, https://doi.org/10.1371/journal.pone.0051374.

Thienemann, A., 1924. Hydrobiologische Untersuchungen an Quellen. *Archiv für Hydrobiologie* 14: 151–189.

Van Der Kamp, G., 1995. The Hydrogeology of Springs in Relation to the Biodiversity of Spring Fauna : A Review. *Journal of the Kansas Entomological Society* 68: 4–17.

Vasquez, A. A., O. Mohiddin, Z. Li, B. L. Bonnici, K. Gurdziel, & J. L. Ram, 2021. Molecular diet studies of water mites reveal prey biodiversity. *PLoS ONE* 16: 1–18, https://doi.org/10.1371/journal.pone.0254598.

Verberk, W. C. E. P., & D. T. Bilton, 2013. Respiratory control in aquatic insects dictates their vulnerability to global warming. *Biology Letters* 9: 0–3, https://doi.org/10.1098/rsbl.2013.0473.

Von Fumetti, S., F. Bieri-Wigger, & P. Nagel, 2017. Temperature variability and its influence on macroinvertebrate assemblages of alpine springs. *Ecohydrology* 10: 1–9, https://doi.org/10.1002/eco.1878.

Von Fumetti, S., & L. Blattner, 2017. Faunistic assemblages of natural springs in different areas in the Swiss National Park: a small-scale comparison. *Hydrobiologia* Springer International Publishing 793: 175–184, https://doi.org/10.1007/s10750-016-2788-5.

Von Fumetti, S., & P. Nagel, 2012. Discharge variability and its effect on faunistic assemblages in springs. *Freshwater Science* 31: 647–656, https://doi.org/10.1899/10-159.1.

Walker, M., S. Johnsen, S. O. Rasmussen, T. Popp, J.-P. Steffensen, P. Gibbard, W. Hoek, J. Lowe, J. Andrews, S. Björck, L. C. Cwynar, K. Hughen, P. Kershaw, B. Kromer, T. Litt, D. J. Lowe, T. Nakagawa, R. Newnham, & J. Schwander, 2009. Formal definition and dating of the GSSP (Global Stratotype Section and Point) for the base of the Holocene using the Greenland NGRIP ice core, and selected auxiliary records. *Journal of Quaternary Science* John Wiley & Sons, Ltd 24: 3–17, https://doi.org/https://doi.org/10.1002/jqs.1227.

Ward, J. V., 1994. Ecology of alpine streams. *Freshwater Biology* 32: 277–294, https://doi.org/10.1111/j.1365-2427.1994.tb01126.x.

Więcek, M., P. Martin, & A. Lipinski, 2013. Water mites as potential long-term bioindicators in formerly drained and rewetted raised bogs. *Ecological Indicators* 34: 332–335, https://doi.org/10.1016/j.ecolind.2013.05.019.

Wigger, F. W., L. Schmidlin, P. Nagel, & S. von Fumetti, 2015. Macroinvertebrate assemblages of natural springs along an altitudinal gradient in the Bernese Alps, Switzerland. *Annales de Limnologie - International Journal of Limnology* 51: 237–247, https://doi.org/10.1051/limn/2015018.

Wood, P. J., J. Gunn, H. Smith, & A. Abas-Kutty, 2005. Flow permanence and macroinvertebrate community diversity within groundwater dominated headwater streams and springs. *Hydrobiologia* 545: 55–64, https://doi.org/10.1007/s10750-005-2213-y.

Wurzbacher, C., A. K. Kreiling, S. Svantesson, S. Van den Wyngaert, E. Larsson, F. Heeger, H. R. Nilsson, & S. Pálsson, 2020. Fungal communities in groundwater springs along the volcanic zone of Iceland. *Inland Waters* Taylor & Francis 10: 418–427, https://doi.org/10.1080/20442041.2019.1689065.

Zawal, A., R. Stryjecki, E. Buczyńska, P. Buczyński, J. Pakulnicka, A. Bańkowska, T. Czernicki, K. Janusz, A. Szlauer-Łukaszewska, & V. Pešić, 2018. Water mites (Acari, Hydrachnidia) of riparian springs in a small lowland river valley: what are the key factors for species distribution?. *PeerJ* 6: e4797, https://doi.org/10.7717/peerj.4797.

Chapter II

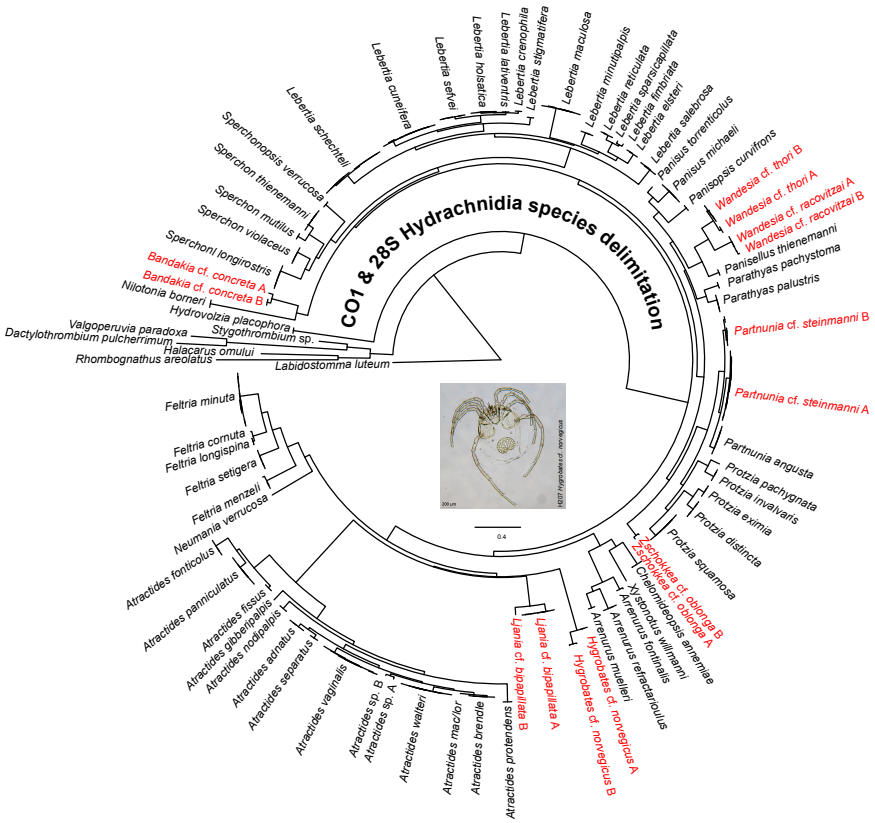
Hidden biodiversity revealed by integrated morphology and genetic species delimitation of spring-dwelling water mite species (Acari, Parasitengona: Hydrachnidia)

Published in *Parasites & Vectors* (2019)

Published article on pages: 23 – 35

The article and accompanying supplementary information are available at:

https://doi.org/10.1186/s13071-019-3750-y



Circular phylogenetic tree of spring-dwelling water mite species. A priori morphospecies that exhibit unexpected intraspecific clade-separation are shown in red color. (Graphical Abstract from Blattner et al. 2019)

Parasites & Vectors

RESEARCH

Open Access

Hidden biodiversity revealed by integrated morphology and genetic species delimitation of spring dwelling water mite species (Acari, Parasitengona: Hydrachnidia)

Lucas Blattner[1*], Reinhard Gerecke[2] and Stefanie von Fumetti[1]

Abstract

Background: Water mites are among the most diverse organisms inhabiting freshwater habitats and are considered as substantial part of the species communities in springs. As parasites, Hydrachnidia influence other invertebrates and play an important role in aquatic ecosystems. In Europe, 137 species are known to appear solely in or near springheads. New species are described frequently, especially with the help of molecular species identification and delimitation methods. The aim of this study was to verify the mainly morphology-based taxonomic knowledge of spring-inhabiting water mites of central Europe and to build a genetic species identification library.

Methods: We sampled 65 crenobiontic species across the central Alps and tested the suitability of mitochondrial (*cox*1) and nuclear (*28S*) markers for species delimitation and identification purposes. To investigate both markers, distance- and phylogeny-based approaches were applied. The presence of a barcoding gap was tested by using the automated barcoding gap discovery tool and intra- and interspecific genetic distances were investigated. Furthermore, we analyzed phylogenetic relationships between different taxonomic levels.

Results: A high degree of hidden diversity was observed. Seven taxa, morphologically identified as *Bandakia concreta* Thor, 1913, *Hygrobates norvegicus* (Thor, 1897), *Ljania bipapillata* Thor, 1898, *Partnunia steinmanni* Walter, 1906, *Wandesia racovitzai* Gledhill, 1970, *Wandesia thori* Schechtel, 1912 and *Zschokkea oblonga* Koenike, 1892, showed high intraspecific *cox*1 distances and each consisted of more than one phylogenetic clade. A clear intraspecific threshold between 5.6–6.0% K2P distance is suitable for species identification purposes. The monophyly of Hydrachnidia and the main superfamilies is evident with different species clearly separated into distinct clades. *cox*1 separates water mite species but is unsuitable for resolving higher taxonomic levels.

Conclusions: Water mite species richness in springs is higher than has been suggested based on morphological species identification alone and further research is needed to evaluate the true diversity. The standard molecular species identification marker *cox*1 can be used to identify species but should be complemented by a nuclear marker, e.g. *28S*, to resolve taxonomic relationships. Our results contribute to the taxonomical knowledge on spring inhabiting Hydrachnida, which is indispensable for the development and implementation of modern environment assessment methods, e.g. metabarcoding, in spring ecology.

Keywords: *cox*1, Barcoding, Species delimitation, Crenobiosis, *28S*, Springs, Biodiversity, Phylogeny, ABGD

*Correspondence: lucas.blattner@unibas.ch
[1] Department of Environmental Sciences, Geoecology Research Group, University of Basel, St. Johanns-Vorstadt 10, 4056 Basel, Switzerland
Full list of author information is available at the end of the article

Background

Water mites (Hydrachnidia) are highly diverse in aquatic habitats [1]. They have a complex life-cycle that includes a prelarva, a parasitic larval stage, an initial resting stage (protonymph), a free living deutonymph, a second resting stage (tritonymph) and the final adult stage [2]. Hydrachnidia disperse predominantly through passive rather than active pathways because water mite larvae parasitize other invertebrate taxa, generally insect hosts that fly [2–5].

Nearly all freshwater environments are inhabited by water mite species with a high degree of habitat specialization [6]. Mites in springs and other groundwater-influenced ecosystems occur in remarkably high diversity of habitats [7–9]. Of the 970 recorded European water mite species, 137 are found solely in or near springs and are adapted to several microhabitats, such as different substrate types and environmental conditions [2, 9]. Due to the high degree of adaptation and their influence on ecosystem functioning for other invertebrate taxa [10–14], these so called crenobiontic (occur exclusively in spring habitats) and crenophilous (tendency to be found in the spring brook) species play a critical role in spring species communities. Considering that springs are island-like habitats within an uninhabitable terrestrial matrix [15, 16], spring dwelling water mite populations are assumed to be rather isolated. This would promote reproductive isolation and therefore lead to an increased speciation rate [4, 17], which is among other things an explanation for the relatively high species diversity of water mites in springs. However, the degree of isolation of spring water mite populations is highly dependent on the dispersal abilities of their hosts and influenced by taxon specific host spectra and specificity [5, 18, 19]. Furthermore, the high microhabitat diversity in springs [15, 20], their relatively stable environmental conditions [21, 22] and absence of large predators, e.g. fish [9], make them exceptionally favorable habitats for insect larvae and benefit their development. Therefore, the diversity and abundance of water mite hosts is relatively high in springs compared to other freshwater habitats, which is likewise considered as precondition for the high number of crenobiont water mite species [9].

Despite their importance for freshwater species communities, the taxonomic knowledge about Hydrachnidia species is still limited today and new species are discovered frequently (e.g. [23–26]). The intensity of re-examination of European Hydrachnidia has increased over the past years with several taxonomic revisions published [9]. Most water mite species known today have been described based on morphology only and studies applying genetic methods to verify and complement these descriptions are still relatively scarce (e.g. [27–30]). Nonetheless, many studies have shown that genetic species delimitation, frequently using *cox*1 barcoding [31], has a large potential to reveal new species, resolve taxonomic questions and contribute to biodiversity baselines and assessments (e.g. [29, 32–35]). Species identifications and their ecological interactions are crucial for contributions to crenobiology, community ecology, developing reliable bioindicators and understanding population dynamics. Moreover, newly developed methods to monitor invertebrate assemblages in freshwater environments, such as the simultaneous identification of bulk sampled individuals (metabarcoding) [36] or the indirect community reconstruction by analyzing environmental DNA (eDNA) [37], rely on previously established genetic species reference databases. Thus, a proper taxonomical knowledge and species description is greatly needed.

Several factors are important to account for when using genetic species identification methods [38], such as the presence of endosymbionts like the alpha-proteobacteria *Wolbachia* sp. [39, 40] or the presence of pseudogenes and nuclear copies of mitochondrial DNA (numts) [41–43], which compromise the suitability of mitochondrial molecular markers to identify species. Standard barcoding methods are mainly based on sequence similarity and the relation between intra- and interspecific genetic distance, which is commonly calculated by using the Kimura 2-parameter (K2P) [44] and uncorrected (p) distances [31, 45, 46]. Nonetheless, the usability of genetic markers to identify species can vary between different taxonomic groups, geographical origin and sampling strategy [31, 38, 47], which implies a taxon-specific evaluation prior to a broad-scale application in environmental assessment and conservation.

In this study we use an integrative taxonomy approach to verify the species status and validity of the most common spring related Hydrachnidia species in Europe. We tested the reliability of techniques commonly used to identify and delimit species using fragments of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1) [31, 48] and the D1-D2 region of the *LSU* rDNA gene (*28S*) [49], or using both (e.g. [28, 50–52]). This study aims at improving knowledge and analytical techniques for assessing Hydrachnidia diversity in springs and explores the strength and weaknesses of standardized barcoding loci to identify water mite species.

Methods

Water mite sampling

The studied crenobiontic and crenophilous Hydrachnidia species were sampled between 2008 and 2018 in 87 different sampling sites across Europe during multiple sampling occasions (Additional file 1: Table S1). Most of the specimens were collected from springs located within the

protected areas Berchtesgaden National Park (Germany), Black Forest National Park (Germany), Gesäuse National Park (Austria) and in the Swiss National Park (Switzerland). Samples were manually collected with a 200-µm hand net. Water mites were either sampled alive in the field or sorted out in the laboratory under a stereomicroscope from mixed samples containing bulk substrate. All specimens were subsequently stored in ethanol (100%) and kept at 4 °C until further processing.

Non-destructive DNA extraction

Total genomic DNA (gDNA) of each individual mite was extracted by using either GeneReleaser® (BioVentures, Murfreesboro, TN, USA) or the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Both methods allow a non-destructive DNA extraction, which is essential when voucher specimens need to be retained for morphological identification in barcoding projects. Prior to both extraction methods, each individual was cleaned by using forceps and entomological needles in a small Petri dish filled with ethanol (100%) under a stereomicroscope. All instruments and vessels used were cleaned after processing each mite specimen by rinsing it with sodium hypochlorite (13%), molecular grade water and ethanol (80%). Afterwards, the specimens were air dried and soaked in molecular grade water for 3 min to ensure the absence of ethanol residues. The cleaned specimen was then transferred to either a 0.2-ml PCR tube containing 0.9 µl of molecular grade water and 0.1 µl of 1× PCR buffer (Qiagen) in the case of GeneReleaser® or to a 1.5-ml tube containing 180 µl of buffer ATL (Qiagen) and 20 µl (20 mg/ml) of Proteinase K (Qiagen) when using the DNeasy Blood & Tissue Kit. The GeneReleaser® method was conducted as originally described by Schizas et al. [53] and modified by Böttger-Schnack & Machida [54], see also [55]. Instead of resuspending the supernatant in TE buffer, step 6 of the modified protocol [54], approximately 12 µl of supernatant was transferred into a new 0.2-ml PCR tube and used directly as DNA template for the subsequent PCR reactions. The DNeasy Blood & Tissue extraction was performed according to the manufacturer's protocol (Animal Tissues, Spin-Column Protocol, Qiagen) with minor changes. The specimens were incubated in buffer ATL and Proteinase K at 56 °C on a shaking thermomixer (400× *rpm*) overnight (step 2 in the manufacturer's protocol) and the elution buffer (AE) volume was decreased to 100 µl in the last step to increase the gDNA concentration. The concentration of every DNA template was measured after the extraction by using a Qbit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the dsDNA HS Assay Kit (Thermo Fisher Scientific). After the first processed specimens it was evident that the mean amount of total gDNA obtained by the DNeasy procedure is higher (mean ± SD: 71.5 ± 2.3 ng in 100 µl of solution, $n = 92$) than when extracting gDNA by using the GeneReleaser® method (mean ± SD: 56.8 ± 4.8 ng in 12 µl of solution, $n = 105$). Therefore, the DNeasy method was chosen for all subsequent extractions.

Morphological examination

All water mite individuals were identified morphologically by the authors RG and LB using current Hydrachnidia identification keys [56–58]. After the DNA extraction, the mite specimens were dissected and mounted on slides in Hoyer's medium or identified as whole individuals under a compound microscope when possible. The enzymatic DNA extraction method (Proteinase K) leads to a partial digestion of the specimens causing discoloration and therefore improved visibility of morphological characters, especially of sclerotized parts. This often allows the morphological identification without dissection. However, digestion is a process that affects membranous parts and therefore deteriorates the observability of integument structures such as the papillae, tubercles or lining. All voucher specimens are stored in the acarological collection of the Natural History Museum Basel (Switzerland) under the museum identifications presented in Additional file 1: Table S1.

PCR amplification and sequencing

The approximate 650 bp standard barcoding fragment of the cytochrome *c* oxidase subunit 1 (*cox*1) [31] mitochondrial gene was first amplified by using universal primers LCO1490 and HCO2198 [59] of a subset of Hydrachnidia species belonging to several genera (*Atractides*, *Feltria*, *Hygrobates*, *Lebertia*, *Partnunia*, *Protzia* and *Sperchon*). PCR reactions contained 0.25 µl of Phusion High Fidelity DNA Polymerase (2 U/µl) (Thermo Fisher Scientific), 5 µl of 5× Phusion HF Buffer (Thermo Fisher Scientific), 0.5 µl of dNTP mix (10 mM) (Sigma-Aldrich, Buchs, SG, Switzerland), 1.25 µl of forward and reverse primers (10 µM each), 5 µl of template DNA and ultrapure water to a total reaction volume of 25 µl. The PCR conditions were as follows: initial denaturation for 30 s at 98 °C; 35 cycles of 10 s at 98 °C, 30 s at 50 °C and 30 s at 72 °C; final elongation for 2 min at 72 °C. PCR products were then stored at 4 °C. As this primer combination failed to amplify several samples we decided to design new genus-specific *cox*1 barcoding primers on the basis of the previously amplified water mite specimens. This was possible due to a low sequence variability at the 5' and 3' ends of the barcoding fragment.

Additionally, a new set of universal barcoding primers was designed by degenerating and modifying several positions of the original LCO1490/HCO2198 primers

to enable a higher amplification performance when barcoding Hydrachnidia species. These new primer sets (Table 1) were used to amplify all remaining samples. The subsequent PCRs were performed by using 0.25 of µl Q5® High-Fidelity DNA Polymerase (2U/µl) (NEB, Ipswich, USA), 5 µl of 5× Q5® Reaction Buffer (NEB), 0.5 µl of dNTP mix (10 mM) (Sigma-Aldrich), 1.25 µl of forward and reverse primers (10 µM each), 5 µl of template DNA and ultrapure water to a total reaction volume of 25 µl. The PCR conditions were the same for all newly designed *cox*1 primer sets and were as follows: initial denaturation for 30 s at 98 °C; 35 cycles of 10 s at 98 °C, 30 s at 51 °C and 20 s at 72 °C; and a final elongation step for 2 min at 72 °C. The PCR products were then kept at 4 °C until further processing. To amplify the D1-D2 domain of the *LSU* rRNA *28S* gene we designed new water mite-specific primer sets on the basis of the D1D2fw2 forward primer [49] and by aligning different *28S* Hydrachnidia sequences downloaded from GenBank. The new primers 28SHy_F and 28SHy_R (Table 1) reliably amplified an approximately 1.2 kbp long fragment of the *28S* D1-D2 domain. The *28S* PCR reactions were done by using the same reaction components and conditions as used when amplifying with the new *cox*1 primer sets. The only difference was a higher annealing temperature at 68 °C instead of 51 °C. All PCR primers (*cox*1 and *28S*) were tailed with modified M13 sequences (M13: 5′-TGT AAA ACG ACG GCC AG-3′ and M13r: 5′-CAG GAA ACA GCT ATG AC-3′) [60], which has shown to improve the amplification and sequencing reactions in this and previous studies [61, 62]. Prior to sequencing, PCR products where examined on an agarose gel electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol on a QIAcube (Qiagen). The purified PCR products were Sanger sequenced with the above mentioned M13 primers by Mycrosynth AG (Balgach, Switzerland).

Molecular analysis

Raw sequences were analyzed, edited and aligned in Geneious Prime v.2019.1.1 [63]. Low-quality base calls, ambiguous sites and primer binding sites at the 5′- and 3′-ends were trimmed prior to further processing. Alignments were done by using MAFFT v.7.388 [64] implemented in Geneious Prime. Potentially poorly aligned positions and divergent regions of the alignments were eliminated with Gblocks v.0.91b [65, 66]. All sequences were tested for the presence of contaminants by blasting with the Nucleotide Blast Tool (BLASTn) implemented on the NCBI website [67]. Because misleading numts can be amplified in PCRs targeting *cox*1 mtDNA, we translated the sequences into amino acids to check for the presence of stop codons, which is commonly seen as a suitable way to detect erroneous amplification [41]. The concatenated alignment containing *cox*1 and *28S* sequences was generated by Sequence Matrix v.1.8 [68]. The suitable nucleotide substitution model for each marker (*cox1*: TPM2uf+I+G4 and *28S*: TVM+I+G4) was selected according to the Bayesian information criterion (BIC) as implemented in ModelTest-NG v.0.1.5 [69], a novel software, which combines features of jModelTest2 [70] and ProtTest3 [71] on the CIPRES Science Gateway v.3.3 [72]. All sequences generated in this study are deposited in NCBI GenBank under the accession numbers MK889511–MK889751 (*cox*1) and MK889752–MK889992 (*28S*) and on BOLDsystems under the IDs LBCWS001-19 to LBCWS245-19 (*cox*1).

Distance-based species delimitation

Intra- and interspecific Kimura 2-parameter (K2P) [44] and uncorrected (p) distances were calculated in MEGA X [73]. The species delimitation threshold was investigated by using the threshold optimization method of the SPIDER (Species Identity and Evolution in R) v.1.5.0 package [74] implemented in R [75] as described in the tutorial (available at: http://spider.r-forge.r-project.org). Additionally, we used the Automated Barcode Gap Discovery (ABGD) procedure [76] to assign the sequences to hypothetical species based on the gap between intra- and interspecific sequence diversity, the so-called "barcoding gap". ABGD was performed on the ABGD web interface [77] by using the MEGA distance files with default parameters, 20 steps and a modified relative gap width of 1. Additionally, the sequences were analyzed by using the Bold Systems v.4 [78, 79] tools available on the Barcode of Life webpage [80].

Phylogenetic species delimitation

Phylogenetic relationships between the sampled Hydrachnidia species were examined with maximum likelihood (ML) and Bayesian inference (BI). RAxML-NG [81], which is a new improved version of RAxML [82], was used to infer the best fitting ML trees of the single markers (*28S* and *cox*1) and the concatenated alignment, respectively. Similar sequences were treated as duplicates and removed automatically by RAxML-NG at the beginning of the tree calculation. ML branch support values were generated by the bootstrap method [83] with 1000 replicates and bipartition support for the best ML tree. Bootstrapping trees were computed directly in RAxML-NG. The BI trees were generated by using the parallel MPI version of MrBayes v.3.2.6 [84, 85]. Bayesian inferences were run for 15×10^6 MCMC generations, sampled every 5000th generation after the exclusion of 25% 'burn-in' by using 4 independent chains. Branches showing

Table 1 Primers designed and used in this study

Marker	Taxon	Name	Direction	Sequence (5′–3′)
*cox*1	Hydrachnidia	LCO_Hydr	F	CAACAAACCAYAAAGAYATTGG
		HCO_Hydr	R	TGGGTGTCCRAARAATCA
	Atractides	Atr_F	F	ACCAYAAAGAYATTGGAAC
		Atr_R	R	AAAATCAGAARATATGTTGA
	Lebertia	Leb_F	F	CAAACCAYAAAGAYATTGGAAC
		Leb_R	R	CGAAGAATCAAAATARRTGTTG
	Partnunia	Part_F	F	ACACTYTACTTYGCTTTTGG
		Part_R	R	CAAAGAATCAAAATAARTGTTG
	Feltria	Fe_F	F	ATATTGGYACTTTATATTTCGG
		Fe_R	R	CGAAGAATCAAAATARATGTTG
	Protzia	Leb_F	F	CAAACCAYAAAGAYATTGGAAC
		Protz_R	R	GATGTRTTAAARTTTCGATCTG
	Hydrovolzia	Hydrov_F	F	TGGGCWGGAATTTTAGGATC
		Hydrov_R	R	TGTTGAAAGAGGATTGGGTC
	Hygrobates	Riv_F	F	CAAACCAYAAAGAYATTGGTAC
		HCO_Hydr	R	TGGGTGTCCRAARAATCA
	Wandesia	Wand_F	F	ACCAYAAAGAYATTGGGACC
		HCO_Hydr	R	TGGGTGTCCRAARAATCA
28S	Hydrachnidia	28SHy_F	F	AGTACCGTGAGGGAAAGTTG
		28SHy_R	R	GGCAGGTGAGTTGTTACACA

Abbreviations: F, forward; R, reverse

bootstrap values below 70% and Bayesian posterior probabilities below 0.95 were interpreted as resolved but not statistically supported [86]. The concatenated alignment was treated as partitioned dataset with unlinked base frequencies, nucleotide substitution rates, gamma shapes and proportions of invariant sites. The rates and frequencies were set according to the ModelTest-NG results. For each marker (*cox*1 and *28S*), the appropriate nucleotide substitution model was used when running RAxML-NG and MrBayes for the single and partitioned analysis, respectively. To resolve the basal nodes and ensure a reliable rooting, several outgroup taxa were added to the tree inferences. The most distant taxon included was the terrestrial mite *Labidostomma luteum* Kramer, 1879 (Labidostommatoidea) (GenBank *28S*/*cox*1: KM100974/GQ864390). Additionally, *Dactylothrombium pulcherrimum* (Haller, 1882) (Trombidioidea) (GenBank: KM100939/KM100985), *Valgoperuvia paradoxa* (Robaux, 1970) (Trombidioidea) (GenBank: KM100943/KM100988) and *Stygothrombium* sp. (Stygotrombidioidea) (GenBank: KM100938/ KM100995) sequences were used as closely related terrestrial Acariformes. The Halacaridae species *Halacarus omului* (Pepato & Da Silveira, 2013) (GenBank: MG751425/MG696236) and *Rhombognathus areolatus* (Abé & Fernandes, 2011) (GenBank: MG751437/MG696244) were chosen as aquatic relatives to the monophyletic Hydrachnidia [87]. The final trees were analyzed and edited in FigTree v.1.4.4 [88], Geneious Prime v.2019.1.1, Dendroscope v3.5.10 [89] and Affinity Designer v.1.6.1 (Serif Europe Ltd., Nottingham, UK).

Results

We successfully amplified and sequenced both target loci (*cox*1 and *28S*, respectively) of 241 individual water mite specimens representing 22 genera and 65 morphologically identified crenobiontic and crenophilous species with 1 to 19 individuals per species (Additional file 1: Table S1). Three individuals belonging to the genus *Atractides* sp. (H450, H528 and H531), all representatives of the *loricatus* species group, were not identifiable to species level. As also observed in other populations of this group collected in various parts of Europe, important diagnostic features, i.e. large *vs* small dorsal muscle attachment sclerites, size of acetabula in the genital field and sclerotized or smooth excretory pore, as well as character state combinations are in disagreement with the identification key in Gerecke et al. [58].

*cox*1 final alignment length was 650 bp, 398 sites were polymorphic (389 parsimony informative) and no alignment gaps were present. The 999 bp *28S* alignment showed 466 polymorphic sites (358 parsimony informative) and 137 gap positions. The translation of the *cox*1 sequences into amino acids did not contain any stop codon positions and blasting the sequences confirmed the absence of contaminations. In a few cases, when using the universal primer pair (LCO1490/HCO2198), we amplified Chironomidae DNA instead of water mite DNA and discovered *Wolbachia* sp. infestation. These specimens were excluded from further analysis.

Distance-based species delimitation and discovery

The mean overall pairwise distances were larger between the *cox*1 sequences (K2P ± SD: 0.29 ± 0.10; p-distance ± SD: 0.24 ± 0.07) compared to *28S* (K2P ± SD: 0.15 ± 0.10; p-distance ± SD: 0.14 ± 0.05). Out of the 65 morphologically identified taxa, 11 were singletons, 7

taxa (*Bandakia concreta* Thor, 1913, *Hygrobates norvegicus* (Thor, 1897), *Ljania bipapillata* Thor, 1898, *Partnunia steinmanni* Walter, 1906, *Wandesia racovitzai* Gledhill, 1970, *Wandesia thori* Schechtel, 1912 and *Zschokkea oblonga* Koenike, 1892) showed exceptionally high intraspecific *cox*1 K2P distances (>0.05) and the majority (47 species) had within species K2P distances between 0 and 0.03 (Fig. 1). *Hygrobates norvegicus* exhibited the largest mean genetic *cox*1 distances within morphologically identified species (K2P ± SD: 0.12 ± 0.08; p-distance ± SD: 0.10 ± 0.07), whereas several species showed mean intraspecific K2P distanced below 0.01 (<1%) (Fig. 1).

The SPIDER threshold optimization procedure analysis, which was conducted after the removal of singleton species and sequences of the seven taxa showing exceptionally high intraspecific variation, revealed an optimal K2P distance threshold at 0.056 (5.6%) and a p-distance threshold at 0.053 (5.3%) for species identification purposes with no false positive and low false negative identifications (9 out of 225 sequences). Assuming that *cox*1 species identification threshold, the individuals belonging to the above-mentioned taxa, which show high intraspecific variation, are likely to represent more than one species. This threshold was confirmed by the ABGD method that indicated a barcoding gap between K2P and p-distances of 0.06 and 0.09 (Additional file 2: Figure S1). ABGD initial partition revealed 69 and the recursive partition 70 groups, which can be seen as equivalent to species. Each of the seven taxa showing high intraspecific distances were split into two separate groups. Additionally, *Lebertia schechteli* Thor, 1913 showed clade separation in the recursive but not the initial partition causing the disparate number of groups between the partitions (Additional files 3 and 4: Figures S2 and S3). This generally confirms the SPIDER results and indicates the presence of more species than *the a priori* identified morphospecies. Contrary to that, *Lebertia crenophila* Viets, 1920, *Lebertia holsatica* Viets, 1920 and *Lebertia lativentris* Viets, 1922 as well as *Atractides macrolaminatus/A. loricatus* and *Atractides brendle* Gerecke, 2003 were grouped together as the same species in both ABGD partitions. The same analyses were conducted for the *28S* dataset. However, neither the ABGD nor the SPIDER method revealed a threshold suitable for species identification purposes. ABGD indicated the absence of a clear barcoding gap for the *28S* locus (Additional file 5: Figure S4) and the threshold optimization implemented in the SPIDER R package indicated high levels of false negative and false positive identifications at different thresholds.

Phylogenetic species delimitation and discovery

The results obtained by the distance-based species delimitations were generally confirmed by the phylogenetic approach. However, in contrast to the combined *28S* and *cox*1 analysis, the single marker datasets alone did not allow to correctly reveal phylogenetic relationships at different taxonomic ranks and the BI trees (Additional files 6 and 7: Trees S1 and S2) showed several unresolved nodes and polytomies compared to the better resolved ML trees (Additional files 8 and 9: Figures S5 and S6). *cox*1 analyses incorrectly clustered higher taxonomic levels (e.g. genus, family and superfamily). For example, the genus *Protzia* Piersing, 1896 was clustered together with *Sperchon* Kramer, 1877 instead of the more closely related *Partnunia* Piersing, 1896 (Additional file 8: Figure S5). *28S* correctly reconstructed higher taxonomic levels but did not allow resolving species relationships in

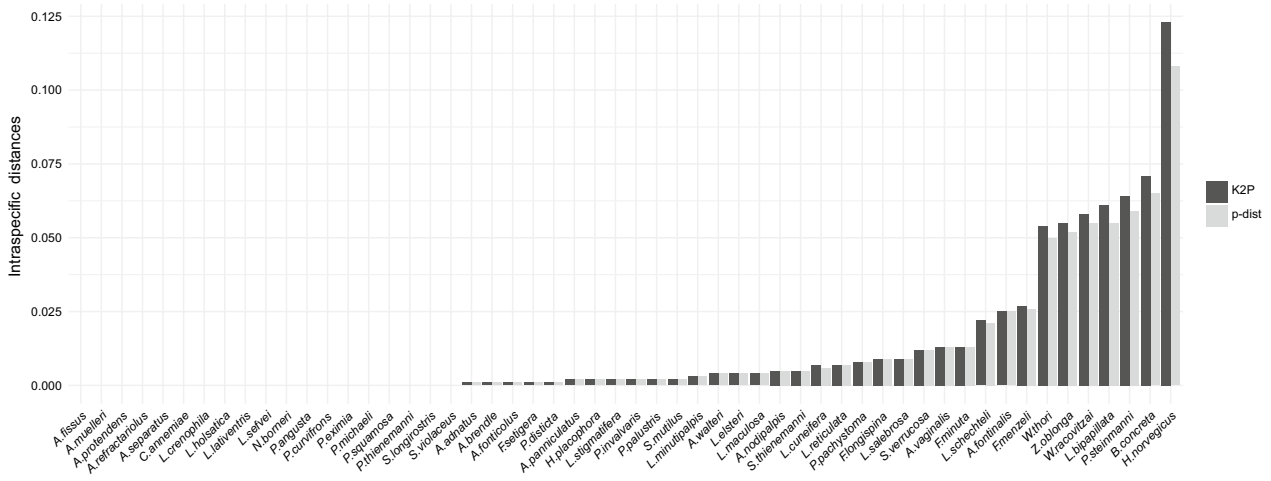


Fig. 1 Intraspecific *cox*1 distances. K2P and p-distances within morphologically identified species represented by more than one individual

several cases, e.g. *Feltria cornuta* Walter, 1927 and *Feltria longispina* Motas & C. Angelier, 1927 or *Lebertia holsatica* Viets, 1920 and *Lebertia lativentris* Viets, 1922 (Additional file 9: Figure S6). Compared to the single marker analysis, the overall taxonomic relatedness was depicted correctly by the combined dataset (Fig. 2). Furthermore, both phylogenetic methods (ML and BI) showed largely congruent and stable tree topologies when applied to the combined *cox*1 and *28S* alignment (Additional file 10: Alignment S1). Due to these findings, we will focus on the ML tree with combined branch support data (Fig. 2).

Results showed that the superfamilies (Hydrovolzioidea, Hydryphantoidea, Lebertioidea, Arrenuroidea and Hygrobatoidea) are monophyletic and clearly separated from each other. A relatively distinct clade affiliation of individuals belonging to the morphologically identified species is evident (Fig. 2). The previously mentioned specimens showing high intraspecific distances are also clearly separated into different clades. All corresponding branches showed high support values, indicating high probabilities of these splits. In the case of *Partnunia steinmanni*, individuals are grouped in two distinct clades, *Partnunia* cf. *steinmanni* A that shares a common ancestor with brook inhabiting (rhithrobiont) *Partnunia angusta* (Koenike, 1893) and *Partnunia* cf. *steinmanni* B. Both morphologically unidentifiable *Atractides* sp. (A & B) individuals represent two genetic species and also the individual belonging to the *Atractides* gr. *macrolaminatus/loricatus* is clearly separated from all other *Atractides* sp. specimens (Fig. 2).

Discussion

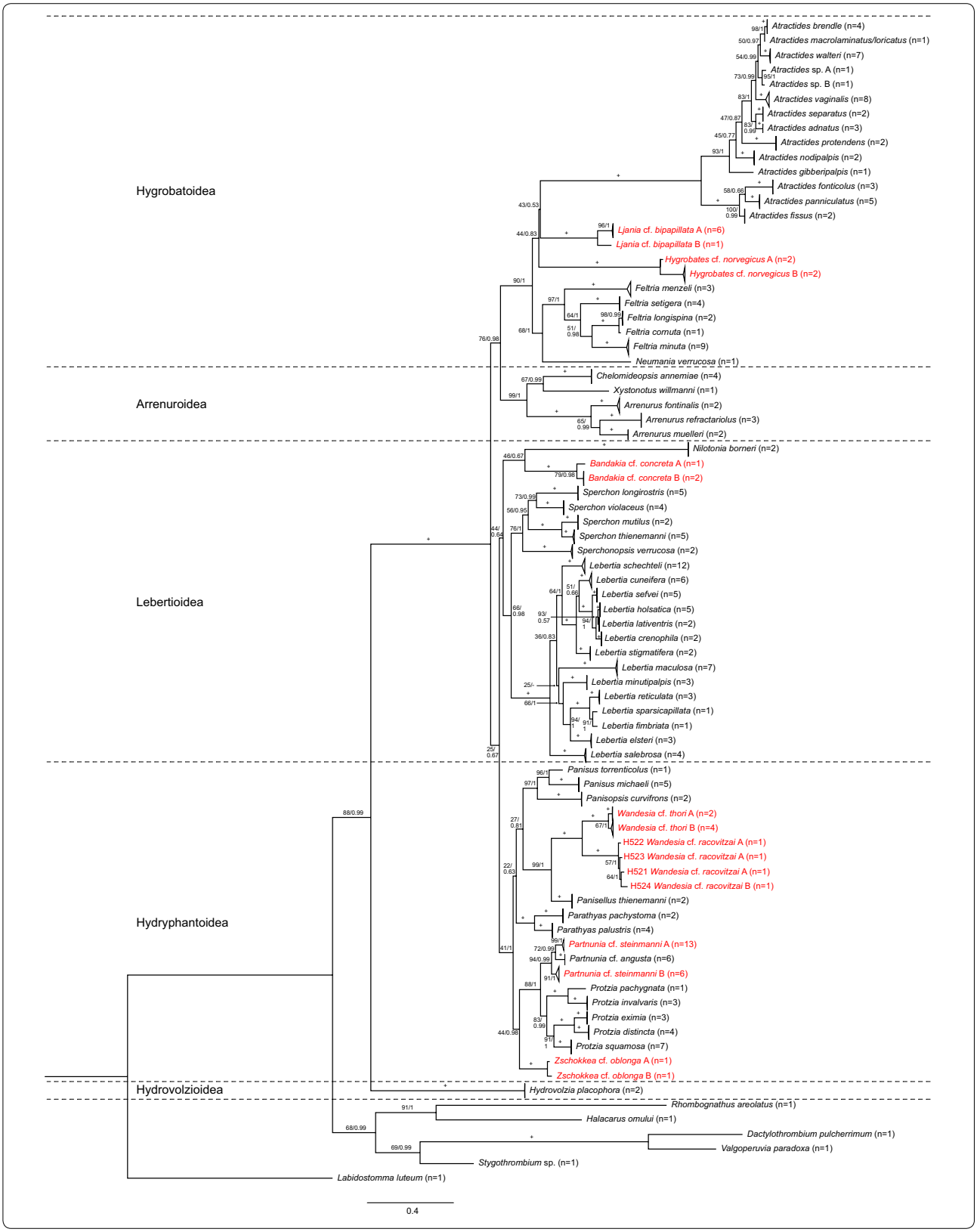
Morphological species identification has a long tradition and is commonly used to identify species for scientific and applied (e.g. ecosystem assessment) purposes [90]. Recently, it has been shown that molecular data reliably complement morphological species identification and has many advantages, especially when used to identify multiple species at once [36, 91]. Furthermore, molecular species delimitation resolves taxonomic uncertainties. When combined with other species-defining characteristics such as morphology it produces a more complete conclusion (e.g. [34, 35, 92, 93]).

Our results generally confirm the morphological species delimitation but show that Hydrachnidia species richness is underestimated and molecular methods are essential to discover currently overlooked biodiversity. The distance-based species delimitation methods revealed an intraspecific *cox*1 threshold between 5.6% (SPIDER) and 6% (ABGD) K2P distance, which is relatively high compared to other taxa [31, 48] but seems to be typical in water mites [28, 29, 94]. However, species delimitation solely based on fixed genetic distance thresholds can be misleading and thresholds should be estimated individually for each dataset [38, 95, 96], especially in taxa with clade-specific intraspecific *cox*1 distances as demonstrated for water mites in this and previous studies (e.g. [94, 97]). As the *28S* marker region did not show a distinct barcoding gap and a clear species identification threshold was not evident, we do not recommend using it as single marker for threshold-based species identification of water mites.

The distance- and morphology-based results were confirmed by applying a phylogenetic approach. A clear monophyletic clade affiliation of individuals belonging to the same species was evident. Both, the distance-based and the phylogeny-based species delimitation revealed that seven morphologically identified species (*Bandakia concreta*, *Hygrobates norvegicus*, *Ljania bipapillata*, *Partnunia steinmanni*, *Wandesia racovitzai*, *Wandesia thori* and *Zschokkea oblonga*) show high genetic differences and therefore are likely to represent more than one species. The *cox*1 ABGD results differed in two cases from the other methods. *Lebertia crenophila*, *L. holsatica* and *L. lativentris* were grouped together as one species as it was also the case in *Atractides macrolaminatus/loricatus* and *A. brendle*. However, these species phylogenetically belong to different clades with high support values and we therefore assume that ABGD erroneously groups the respective sequences and underestimates the number of species, a tendency that has already been shown by other studies (e.g. [98]). Furthermore, ABGD initial and the recursive partition differently grouped *L. schechteli* individuals either as one single or two separate species. The initial partition is considered as more stable and usually better represents the groups that are defined by taxonomists [76, 99]. In addition, the phylogenetic inferences revealed a distinct *L. schechteli* clade. Therefore, we assume that our *L. schechteli* specimens belong to a single species. However, the recursive partition results show

(See figure on next page.)

Fig. 2 Maximum likelihood tree of the combined *cox*1 and *28S* datasets. Support values are shown as bootstrap (BS) and posterior probability (PP) values (PP/BS). Nodes fully supported by PP = 1 and BS = 100 are shown as +. In the case of clear monophyletic clades, tip nodes with more than one individual were collapsed with the number of individuals indicated as (*n* = X). In the case of clade separation within morphologically identified species we named the corresponding specimens by using the species name, cf. and A & B. Clades containing more molecular species than the *a priori* morphospecies are marked in red. Specimen IDs and sampling data of the individuals belonging to species are provided in Additional file 1: Table S1


Hygrobatoidea
Arrenuroidea
Lebertioidea
Hydryphantoidea
Hydrovolzioidea
Atractides brendle (n=4)
Atractides macrolaminatus/loricatus (n=1)
Atractides walteri (n=7)
Atractides sp. A (n=1)
Atractides sp. B (n=1)
Atractides vaginalis (n=8)
Atractides separatus (n=2)
Atractides adnatus (n=3)
Atractides protendens (n=2)
Atractides nodipalpis (n=2)
Atractides gibberipalpis (n=1)
Atractides fonticolus (n=3)
Atractides panniculatus (n=5)
Atractides fissus (n=2)
Ljania cf. bipapillata A (n=6)
Ljania cf. bipapillata B (n=1)
Hygrobates cf. norvegicus A (n=2)
Hygrobates cf. norvegicus B (n=2)
Feltria menzeli (n=3)
Feltria setigera (n=4)
Feltria longispina (n=2)
Feltria cornuta (n=1)
Feltria minuta (n=9)
Neumania verrucosa (n=1)
Chelomideopsis annemiae (n=4)
Xystonotus willmanni (n=1)
Arrenurus fontinalis (n=2)
Arrenurus refractariolus (n=3)
Arrenurus muelleri (n=2)
Nilotonia borneri (n=2)
Bandakia cf. concreta A (n=1)
Bandakia cf. concreta B (n=2)
Sperchon longirostris (n=5)
Sperchon violaceus (n=4)
Sperchon mutilus (n=2)
Sperchon thienemanni (n=5)
Sperchonopsis verrucosa (n=2)
Lebertia schechteli (n=12)
Lebertia cuneifera (n=6)
Lebertia sefvei (n=5)
Lebertia holsatica (n=5)
Lebertia lativentris (n=2)
Lebertia crenophila (n=2)
Lebertia stigmatifera (n=2)
Lebertia maculosa (n=7)
Lebertia minutipalpis (n=3)
Lebertia reticulata (n=3)
Lebertia sparsicapillata (n=1)
Lebertia fimbriata (n=1)
Lebertia elsteri (n=3)
Lebertia salebrosa (n=4)
Panisus torrenticolus (n=1)
Panisus michaeli (n=5)
Panisopsis curvifrons (n=2)
Wandesia cf. thori A (n=2)
Wandesia cf. thori B (n=4)
H522 Wandesia cf. racovitzai A (n=1)
H523 Wandesia cf. racovitzai A (n=1)
H521 Wandesia cf. racovitzai A (n=1)
H524 Wandesia cf. racovitzai B (n=1)
Panisellus thienemanni (n=2)
Parathyas pachystoma (n=2)
Parathyas palustris (n=4)
Partnunia cf. steinmanni A (n=13)
Partnunia cf. angusta (n=6)
Partnunia cf. steinmanni B (n=6)
Protzia pachygnata (n=1)
Protzia invalvaris (n=3)
Protzia eximia (n=3)
Protzia distincta (n=4)
Protzia squamosa (n=7)
Zschokkea cf. oblonga A (n=1)
Zschokkea cf. oblonga B (n=1)
Hydrovolzia placophora (n=2)
Rhombognathus areolatus (n=1)
Halacarus omului (n=1)
Dactylothrombium pulcherrimum (n=1)
Valgoperuvia paradoxa (n=1)
Stygothrombium sp. (n=1)
Labidostomma luteum (n=1)
0.4

the possibility of differentiation and indicate that further research is advisable. In the analysis of *H. norvegicus* and *P. steinmanni* we were able to include several individuals per genotype, whereas in the other taxa differentiation is based on single individuals. Therefore, further individuals of different populations will be processed prior to the final description of the potentially new species.

Results suggest the monophyly of Hydrachnidia and the previously defined superfamilies, supporting the findings of Dabert et al. [87]. In contrast to the latter authors, and possibly due to the inclusion of more taxa in this study, our data support a Hydryphantoidea monophylum. However, the corresponding node separating Hydryphantoidea and Lebertioidea is poorly supported in our results and therefore should be further questioned. Similar to the study of Dabert et al. [87], we equally observed that *cox*1 or *28S* alone are incapable of fully resolving phylogenetic relationships. This phenomenon can probably be caused by mito-nuclear discordance, which has already been recorded for mites and other taxa [100, 101] and was reviewed intensively [102]. Besides this assumption postulating diverging nuclear and mitochondrial phylogenies, the different taxonomic resolution of the two marker regions is likely caused by the loss of phylogenetic information. This can be explained by the fast-evolving character of mitochondrial compared to nuclear DNA and therefore faster loss of ancestral polymorphisms in *cox*1 [102–105]. Arabi et al. [105] showed exceptionally high mitogenomic rearrangements especially in Chelicerata, which fosters this assumption. Therefore, we conclude the necessity of complementing the standard barcoding marker *cox*1 with at least one additional genetic marker, e.g. *28S* or *18S* rDNA, to investigate species relationships and fully resolve water mite taxonomy.

These findings show that the choice of species identification markers must be done with caution and should be adjusted to the research question. For Hydrachnidia, *cox*1 serves as a useful marker if solely species identification is of interest. If the goal is to assign sequences to higher taxonomic levels, another locus should be taken into account. This is also crucial if the aim is to assess the amount of undescribed species in an environment. Currently, most metabarcoding approaches are based on *cox*1 alone [106–109] and few studies investigated the performance of alternative loci (e.g. [110–112]). Apart from the taxon assignment limitations when using *cox*1 alone, our findings show that the primer bias problem [36, 111] needs to be considered when water mites are targeted in metabarcoding studies as universal *cox*1 primers show unsatisfactory amplification performance. This could potentially be circumvented by using a combination of more specific *cox*1 primer sets as has been done in this study, a better matching universal one or the establishment of a new metabarcoding marker for this taxon. We were able to show that molecular methods have a great potential to reveal new water mite species and more studies are needed to complete barcoding databases and refine biodiversity estimates. Fundamental knowledge of species diversity is an essential precondition for implementing water mites in recent monitoring approaches and use them as powerful bioindicators [113, 114] in freshwater assessments as for example required by the European Water Framework Directive (WFD) [115], and may also offer an opportunity for a more nuanced understanding of environmental change impacts on springs systems.

Looking at spring inhabiting Hydrachnidia species, our work contributes to the accumulation of species barcoding data. Our data covers 47.5% of the currently described 137 spring water mite species in Europe [9] and covers the most abundant taxa, especially in the central Alps. Compared to other studies, which investigate Hydrachnidia diversity by applying morphological and molecular techniques in other aquatic habitats than springs (e.g. [28, 87, 94, 116, 117]), we were able to include a large subset of different species belonging to different taxonomic groups including the Proto-, Eu- and Neohydrachnidia [87]. A relatively high proportion of morphologically identified species (10.8%) showed to be more diversified than had been assumed, which indicates an overall underestimation of Hydrachnidia species richness in springs and other aquatic habitats. This indicates that species diversity related research questions such as host specificity of different water mite species need to be reconsidered. *Hygrobates norvegicus*, *Partnunia steinmanni* and *Ljania bipapillata* were shown to have a rather wide host species spectrum [18, 19]. Considering our results, which indicate that these three morphologically described taxa consist of several novel species, the number of hosts per water mite species could be lower and reveal a tendency towards high host specificity.

The dispersal abilities of Hydrachnidia highly depend on the parasitic larval stage that attaches to an insect host that can carry it to a different habitat and therefore governs water mite presence or absence in an environment [2, 13, 118]. Especially in rather isolated freshwater habitats like springs, water mite species dispersal is directly linked with their specific hosts leading to the conclusion that phylogeographic patterns are shared between hosts and parasites. Combined with our assumption of increased host specificity due to the unexpected high degree of Hydrachnidia species diversification, future studies on gene flow patterns between water mite

populations can verify the hypothesis that springs are isolated island-like habitats for the mites as well as their insect hosts.

In Europe, 970 water mite species have been recorded to date [9] and, applying our findings, at least 105 additional species potentially exist. Due to the fact that water mite species diversity increases towards southern parts of Europe [9] we expect an even higher degree of undiscovered species as our dataset mainly consists of specimens collected in central Europe. On this basis, future water mite barcoding projects will be able to gradually fill the gaps of taxonomic knowledge. This is an important prerequisite to incorporating modern species identification and monitoring techniques (e.g. metabarcoding) in future water mite-related freshwater and spring assessment studies.

Conclusions

Our aim was to verify and complement the mainly morphology-based species delimitation of an often neglected, highly diverse taxon in freshwater ecosystems. Our results show that water mite diversity in springs seems to be higher than expected. Molecular methods are largely congruent with morphology and serve as a species delimitation and identification tool. They are particularly powerful if species discovery is the main goal. *cox*1 as a standard barcoding marker is useful for identifying Hydrachnidia species but is not suitable for assigning them to higher taxonomic levels (e.g. genera, families or superfamilies). This limitation can be overcome by using distance- and phylogeny-based multi marker approaches. Our data contributes to genetic species identification databases by adding crenobiontic water mite sequences, which is a precondition for implementing modern methods of freshwater ecosystem assessment such as metabarcoding and eDNA species community monitoring in spring ecology.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-019-3750-y.

Additional file 1: Table S1. List of specimens with sampling data, voucher museum IDs (Natural History Museum of Basel) and the sequence accession numbers.

Additional file 2: Figure S1. ABGD output plots of the *cox*1 K2P (A&B) and p-distances (C&D).

Additional file 3: Figure S2. Initial partition ABGD *cox*1 output tree. Shows individuals grouped as putative species delimited by the ABGD method. Clades indicating more species than the a-priori morphospecies are marked in red.

Additional file 4: Figure S3. Recursive partition ABGD *cox*1 output tree. Shows individuals grouped as putative species delimited by the ABGD method. Clades indicating more species than the a-priori morphospecies are marked in red and differences to the initial partition in blue.

Additional file 5: Figure S4. ABGD output plots of the *28S* K2P (A&B) and p-distances (C&D).

Additional file 6: Tree S1. *cox*1 phylogenetic BI tree MrBayes output file containing support values (posterior probability) (can be viewed in the program FigTree).

Additional file 7: Tree S2. *28S* phylogenetic BI tree MrBayes output file containing support values (posterior probability) (can be viewed in the program FigTree).

Additional file 8: Figure S5. *cox*1 RAxML-NG Maximum Likelihood tree with support values. Clades indicating more species than the a-priori morphospecies are marked in red.

Additional file 9: Figure S6. *28S* RAxML-NG Maximum Likelihood tree with support values. Clades indicating more species than the a-priori morphospecies are marked in red.

Additional file 10: Alignment S1. MAFFT Alignment of the combined (*cox*1 and *28S*) dataset and the root GenBank sequences, used to generate the phylogenetic tree.

Abbreviations

28S: large subunit ribosomal RNA gene; *18S*: small subunit ribosomal RNA gene; ABGD: Automatic Barcode Gap Discovery (species delimitation algorithm); BI: Bayesian inference (phylogenetic tree inference method); BIC: Bayesian information criterion; BLASTn: Nucleotide Basic Local Alignment Search Tool; BOLD: Barcode of Life Database; BS: bootstrap; *cox*1: cytochrome *c* oxidase subunit 1 gene; eDNA: environmental DNA; G: Gamma parameter; gDNA: genomic DNA; I: invariant sites; K2P: Kimura 2-parameter; LSU: large subunit; MAFFT: multiple alignment using fast Fourier transform (sequence alignment algorithm); MCMC: Markov chain Monte Carlo; MEGA: Molecular Evolutionary Genetic Analysis (molecular genetics software); ML: maximum likelihood (phylogenetic tree inference method); MPI: message passing interface; numts: nuclear copies of mitochondrial DNA; PP: posterior probability; RAxML: randomized accelerated maximum likelihood (phylogenetic tree inference software); rDNA: ribosomal DNA; SPIDER: Species Identity and Evolution in R; WFD: Water Framework Directive.

Acknowledgements

We thank numerous persons from the Berchtesgaden (Germany) and Gesäuse (Austria) National Parks as well as the Swiss National Park for support and assistance during the fieldwork. Special thanks go to Nathanael Beck for his advice and helpful comments on the molecular techniques. We appreciate the commitment and cooperation of Alicia Escher and Enrica Steiner for data collection. Furthermore, we thank Dr Colin J. Courtney Mustaphi and Joshua Ebner for language improvement and helpful comments. We appreciate the suggestions and comments of the anonymous reviewers and the editor, who greatly helped to improve the manuscript.

Authors' contributions

All authors contributed to the study design. LB and RG carried out sample collection and the morphological identification of the Hydrachnidia specimens. LB performed all laboratory work, analyzed data, conducted bioinformatics and wrote the manuscript. All authors read and approved the final manuscript.

Funding

This study was made possible by funding from the Swiss National Science Foundation (SNSF) within the project: "Adaptation or isolation: Disenthralling the myth of crenobiosis of spring-dwelling invertebrates" (grant no. 31003A_176234 to SvF).

Availability of data and materials

Data analyzed during this study are included in this published article and its additional files. New sequences generated in this work were deposited in the GenBank database under the accession numbers MK889511–MK889751

(*cox*1) and MK889752–MK889992 (*28S*) and on BOLDsystems under the IDs LBCWS001-19 to LBCWS245-19 (*cox*1). Water mite voucher specimens are stored in the acarological collection of the Natural History Museum Basel (Switzerland) under the identifications NMB-TROM-10000 to NMB-TROM-10240.

Ethics approval and consent to participate
Not applicable.

Consent of publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Department of Environmental Sciences, Geoecology Research Group, University of Basel, St. Johanns-Vorstadt 10, 4056 Basel, Switzerland. [2] Department of Biology, University of Tübingen, Auf der Morgenstelle 28E, 72076 Tübingen, Germany.

Received: 13 May 2019 Accepted: 12 October 2019
Published online: 21 October 2019

References

1. Di Sabatino A, Smit H, Gerecke R, Goldschmidt T, Matsumoto N, Cicolani B. Global diversity of water mites (Acari, Hydrachnidia; Arachnida) in freshwater. Hydrobiologia. 2008;595:303–15.
2. Di Sabatino A, Gerecke R, Martin P. The biology and ecology of lotic water mites (Hydrachnidia). Freshw Biol. 2000;44:47–62.
3. Bohonak AJ. Effect of insect-mediated dispersal on the genetic structure of postglacial water mite populations. Heredity. 1999;82:451–61.
4. Bilton DT, Freeland JR, Okamura B. Dispersal in freshwater invertebrates. Annu Rev Ecol Syst. 2001;32:159–81.
5. Zawal A. The role of insects in the dispersion of water mites. Acta Biol Univ Daugavp. 2003;3:9–14.
6. Walter DE, Proctor HC. Mites: ecology, evolution & behaviour. 2nd ed. Dodrecht: Springer; 2013.
7. Stoch F, Gerecke R, Pieri V, Rossetti G, Sambugar B. Exploring species distribution of spring meiofauna (Annelida, Acari, Crustacea) in the south-eastern Alps. J Limnol. 2011;70:65–76.
8. Di Sabatino A, Cicolani B, Gerecke R. Biodiversity and distribution of water mites (Acari, Hydrachnidia) in spring habitats. Freshw Biol. 2003;48:2163–73.
9. Gerecke R, Martin P, Gledhill T. Water mites (Acari: Parasitengona: Hydrachnidia) as inhabitants of groundwater-influenced habitats - considerations following an update of Limnofauna Europaea. Limnologica. 2018;69:81–93.
10. Lanciani C. The influence of parasitic water mites on the instantaneous death rate of their hosts. Oecologia. 1979;44:60–2.
11. Smith BP. Host-parasite interaction and impact of larval water mites on insects. Ann Rev Entomol. 1988;33:487–507.
12. Smith IM. Water mites (Acari: Parasitengona: Hydrachnidia) of spring habitats in Canada. Mem Entomol Soc Canada. 1991;155:141–67.
13. Martin P, Gerecke R. Diptera as hosts of water mite larvae—an interesting relationship with many open questions tionship with many open questions. Lauterbornia. 2009;68:95–103.
14. Werblow A, Martin P, Dörge DD, Koch LK, Mehlhorn H, Melaun C. Hyperparasitism of mosquitoes by water mite larvae. Parasitol Res. 2015;114:2757–65.
15. Cantonati M, Gerecke R, Bertuzzi E. Springs of the Alps—sensitive ecosystems to environmental change: from biodiversity assessments to long-term studies. Hydrobiologia. 2006;562:59–96.
16. Von Fumetti S, Blattner L. Faunistic assemblages of natural springs in different areas in the Swiss National Park: a small-scale comparison. Hydrobiologia. 2016;793:175–84.
17. Rabosky DL. Reproductive isolation and the causes of speciation rate variation in nature. Biol J Linn Soc. 2016;118:13–25.
18. Martin P, Stur E, Wiedenbrug S. Larval parasitism of spring-dwelling alpine water mites (Hydrachnidia, Acari): a study with particular reference to chironomid hosts. Aquat Ecol. 2009;44:431–48.
19. Martin P, Stur E. Parasite-host associations and life cycles of spring-living water mites (Hydrachnidia, Acari) from Luxembourg. Hydrobiologia. 2006;573:17–37.
20. Gerecke R, Di Sabatino A. Water mites (Hydrachnidia and Halacaridae) in spring habitats: a taxonomical and ecological perspective. In: Cantonati M, Bertruzzi E, Spitale D, editors. The Spring habitat: biota and sampling methods. Trento: Museo Tridentino di Scienze Naturali; 2007. p. 193–216.
21. Rosati M, Cantonati M, Primicerio R, Rossetti G. Biogeography and relevant ecological drivers in spring habitats: a review on ostracods of the Western Palearctic. Int Rev Hydrobiol. 2014;99:409–24.
22. Cantonati M, Füreder L, Gerecke R, Jüttner I, Cox EJ. Crenic habitats, hotspots for freshwater biodiversity conservation: toward an understanding of their ecology. Freshw Sci. 2012;31:463–80.
23. Pešić V, Valdecasas AG, García-Jiménez R. Simultaneous evidence for a new species of *Torrenticola* Piersing, 1896 (Acari: Hydrachnidia) from Montenegro. Zootaxa. 2012;3515:38–50.
24. Ding JH, Sun JL, Zhang X. A new species of the water mite genus *Sperchon* Kramer, 1877 from China, with identifying *Sperchon rostratus* lundblad, 1969 through DNA barcoding (Acari, Hydrachnidia, Sperchontidae). ZooKeys. 2017;2017:47–61.
25. Pešić V, Smit H. A checklist of water mites of Central Asia with description of six new species (Acari, Hydrachnidia) from Kyrgyzstan. Acarologia. 2018;58:165–85.
26. Smit H. A second species of the water mite genus Sinhaladwipabates Gledhill & Wiles, 1997 from Thailand (Acari: Hydrachnidia: Hygrobatidae). Ecol Montenegrina. 2018;101:99–101.
27. Asadi M, Hinomoto N, Saboori A, Javan-Nikkhah M. Genetic diversity in mitochondrial cytochrome *c* oxidase subunit I sequences of the water mite *Hygrobates fluviatilis* (Acari: Hydrachnidia: Hygrobatidae). Int J Acarol. 2012;38:96–100.
28. Staalstedt J, Bergsten J, Ronquist F. "Forms" of water mites (Acari: Hydrachnidia): intraspecific variation or valid species? Ecol Evol. 2013;3:3415–35.
29. Pešić V, Asadi M, Cimpean M, Dabert M, Esen Y, Gerecke R, et al. Six species in one: evidence of cryptic speciation in the *Hygrobates fluviatilis* complex (Acariformes, Hydrachnidia, Hygrobatidae). Syst Appl Acarol. 2017;22:1327–37.
30. Pešić V, Broda Ł, Dabert M, Gerecke R, Martin P, Smit H. Re-established after hundred years: definition of Hygrobates prosiliens Koenike, 1915, based on molecular and morphological evidence, and redescription of H. longipalpis (Hermann, 1804) (Acariformes, Hydrachnidia, Hygrobatidae). Syst Appl Acarology. 2019;24:1490–511.
31. Hebert PDN, Cywinska A, Ball SL, DeWaard JR. Biological identifications through DNA barcodes. Proc Biol Sci. 2003;270:313–21.
32. Copilaș-Ciocianu D, Zimța A-A, Petrusek A. Integrative taxonomy reveals a new *Gammarus* species (Crustacea, Amphipoda) surviving in a previously unknown southeast European glacial refugium. J Zool Syst Evol Res. 2018;57:272–97.
33. Lin X-L, Stur E, Ekrem T. DNA barcodes and morphology reveal unrecognized species in Chironomidae (Diptera). Insect Syst Evol. 2017;49:329–98.
34. Weiss M, Macher JN, Seefeldt MA, Leese F. Molecular evidence for further overlooked species within the *Gammarus fossarum* complex (Crustacea: Amphipoda). Hydrobiologia. 2014;721:165–84.
35. Montagna M, Mereghetti V, Lencioni V, Rossaro B. Integrated taxonomy and DNA barcoding of alpine midges (Diptera: Chironomidae). PLoS ONE. 2016;11:e0149673.
36. Elbrecht V, Vamos EE, Meissner K, Aroviita J, Leese F. Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. Methods Ecol Evol. 2017;8:1265–75.
37. Mächler E, Deiner K, Steinmann P, Altermatt F. Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. Freshw Sci. 2014;33:1174–83.
38. Meyer CP, Paulay G. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 2005;3:e422.

39. Klopfstein S, Kropf C, Baur H. *Wolbachia* endosymbionts distort DNA barcoding in the parasitoid wasp genus *Diplazon* (Hymenoptera: Ichneumonidae). Zool J Linn Soc. 2016;177:541–57.
40. Smith MA, Bertrand C, Crosby K, Eveleigh ES, Fernandez-Triana J, Fisher BL. *Wolbachia* and DNA barcoding insects: patterns, potential, and problems. PLoS ONE. 2012;7:e36514.
41. Song H, Buhay JE, Whiting MF, Crandall KA. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proc Natl Acad Sci USA. 2008;105:13486–91.
42. Haran J, Koutroumpa F, Magnoux E, Roques A, Roux G. Ghost mtDNA haplotypes generated by fortuitous NUMTs can deeply disturb infra-specific genetic diversity and phylogeographic pattern. J Zool Syst Evol Res. 2015;53:109–15.
43. Hazkani-Covo E, Zeller RM, Martin W. Molecular poltergeists: mitochondrial DNA copies (numts) in sequenced nuclear genomes. PLoS Genet. 2010;6:e1000834.
44. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980;16(2):111–20.
45. Jinbo U, Kato T, Ito M. Current progress in DNA barcoding and future implications for entomology. Entomol Sci. 2011;14:107–24.
46. Collins RA, Boykin LM, Cruickshank RH, Armstrong KF. Barcoding's next top model: an evaluation of nucleotide substitution models for specimen identification. Methods Ecol Evol. 2012;3:457–65.
47. Waugh J. DNA barcoding in animal species: progress, potential and pitfalls. BioEssays. 2007;29:188–97.
48. Hebert PD, Ratnasingham S, DeWaard JR. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc R Soc London Ser B Biol Sci. 2003;270:S96.
49. Sonnenberg R, Nolte A, Tautz D. An evaluation of *LSU* rDNA D1-D2 sequences for their use in species identification. Front Zool. 2007;4:6.
50. Lehmitz R, Decker P. The nuclear 28S gene fragment D3 as species marker in oribatid mites (Acari, Oribatida) from German peatlands. Exp Appl Acarol. 2017;71:259–76.
51. Mironov SV, Dabert J, Dabert M. A new feather mite species of the genus *Proctophyllodes* Robin, 1877 (Astigmata: Proctophyllodidae) from the long-tailed tit *Aegithalos caudatus* (Passeriformes: Aegithalidae)-morphological description with DNA barcode data. Zootaxa. 2012;61:54–61.
52. Vasquez AA, Qazazi MS, Fisher JR, Failla AJ, Rama S, Ram JL. New molecular barcodes of water mites (Trombidiformes: Hydrachnidiae) from the Toledo Harbor region of Western Lake Erie, USA, with first barcodes for *Krendowskia* (Krendowskiidae) and *Koenikea* (Unionicolidae). Int J Acarol. 2017;43:494–8.
53. Schizas NV, Street GT, Coull BC, Chandler GT, Quattro JM. An efficient DNA extraction method for small metazoans. Mol Mar Biol Biotechnol. 1997;6:381–3.
54. Böttger-Schnack R, Machida RJ. Comparison of morphological and molecular traits for species identification and taxonomic grouping of oncaeid copepods. Hydrobiologia. 2011;666:111–25.
55. Weigand AM. New *Zospeum* species (Gastropoda, Ellobioidea, Carychiidae) from 980 m depth in the Lukina Jama-Trojama cave system (Velebit Mts., Croatia). Subteranean Biol. 2013;11:45–53.
56. Bartsch I, Davids C, Deichsel R, Di Sapatino A, Gabrys G, Gerecke R. Chelicerata: Araneae, Acari I. In: Gerecke R, editor. Süsswasserfauna von Mitteleuropa. Heidelberg: Spektrum Akademischer Verlag; 2007.
57. Di Sabatino A, Gerecke R, Gledhill T, Smit H. Chelicerata: acari II. In: Gerecke R, editor. Süsswasserfauna von Mitteleuropa. Heidelberg: Spektrum Akademischer Verlag; 2010. p. 234.
58. Gerecke R, Gledhill T, Pešić V, Smit H. Süßwasserfauna Von Mitteleuropa, Bd 7/2-3. Chelicerata: Acari III. Heidelberg: Springer; 2016.
59. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.
60. Messing J. New M13 vectors for cloning. Methods Enzymol. 1983;101:20–78.
61. Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN. Universal primer cocktails for fish DNA barcoding. Mol Ecol Notes. 2007;7:544–8.
62. Kress WJ, Erickson DL. DNA barcodes: methods and protocols. In: Barcodes DNA, editor. Humana Press. Totowa: NJ; 2012. p. 3–8.
63. Geneious. Geneious Prime. 2019. https://www.geneious.com. Accessed 13 Aug 2019.
64. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30:772–80.
65. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol. 2007;56:564–77.
66. Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol. 2000;17:540–52.
67. NCBI. BLASTn. 2019. https://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed 22 Aug 2019.
68. Vaidya G, Lohman DJ, Meier R. SeqenceMatrix: cladistics multi-gene datasets with character set and codon information. Cladistics. 2011;27:171–80.
69. Darriba D, Posada D. ModelTest-NG. 2015. https://github.com/ddarriba/modeltest. Accessed 11 Feb 2019.
70. Darriba D, Taboada GL, Doallo R, Posada D. JModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012;9:772.
71. Darriba D, Taboada GL, Doallo R, Posada D. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics. 2011;27:1164–5.
72. Miller MA, Pfeiffer W, Schwartz T. Creating the CIPRES science gateway for inference of large phylogenetic trees. In: Proceedings of the gateway computing environments workshop (GCE). New Orleans; 2010. p. 1–8.
73. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9.
74. Boyer S, Brown SDJ, Malumbres-Olarte J, Vink CJ, Cruickshank RH, Collins RA, et al. Spider: an R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. Mol Ecol Resour. 2012;12:562–5.
75. R Core Team. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2017.
76. Puillandre N, Lambert A, Brouillet S, Achaz G. ABGD, automatic barcode gap discovery for primary species delimitation. Mol Ecol. 2012;21:1864–77.
77. Achaz G. ABGD HomePage. 2019. http://wwwabi.snv.jussieu.fr/public/abgd/. Accessed 20 Aug 2019.
78. Ratnasingham S, Hebert PDN. The Barcode of Life Data System (http://www.barcodinglife.org). Mol Ecol Notes. 2007;7:355–64.
79. Ratnasingham S, Hebert PDN. A DNA-based registry for all animal species: the Barcode Index Number (BIN) system. PLoS ONE. 2013;8:e66213.
80. Barcode of Life Data Systems. BOLDsystems v4. 2019. http://boldsystems.org. Accessed 12 Jul 2019.
81. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics. 2019. https://doi.org/10.1093/bioinformatics/btz305.
82. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–3.
83. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985;39:783–91.
84. Darling A, Ronquist F, Ayres DL, Larget B, Liu L, Teslenko M. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012;61:539–42.
85. Altekar G, Dwarkadas S, Huelsenbeck JP, Ronquist F. Parallel Metropolis coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. Bioinformatics. 2004;20:407–15.
86. Douady CJ, Delsuc F, Boucher Y, Doolittle WF, Douzery EJP. Comparison of Bayesian and maximum likelihood bootstrap measures of phylogenetic reliability. Mol Biol Evol. 2003;20:248–54.
87. Dabert M, Proctor H, Dabert J. Higher-level molecular phylogeny of the water mites (Acariformes: Prostigmata: Parasitengonina: Hydrachnidiae). Mol Phylogenet Evol. 2016;101:75–90.
88. Rambaut A. FigTree. 2019. http://tree.bio.ed.ac.uk/software/figtree. Accessed 12 Jul 2019.
89. Huson DH, Scornavacca C. Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. Syst Biol. 2012;61:1061–7.
90. Hebert PDN, Gregory TR. The promise of DNA barcoding for taxonomy. Syst Biol. 2005;54:852–9.

91. Baird DJ, Hajibabaei M. Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. Mol Ecol. 2012;21:2039–44.
92. De Rojas M, Doña J, Jovani R, Dimov I, Zurita A, Callejón R. Evidence of cryptic species in the genus *Tinaminyssus* (Acari: Rhinonyssidae) based on morphometrical and molecular data. Exp Appl Acarol. 2018;75:355–68.
93. Stryjecki R, Bańkowska A, Gryzińska M, Sarnacka E, Rutkowska M, Zawal A. The use of molecular techniques in the taxonomy of water mites (Hydrachnidia, Acari). Acta Biol. 2016;23:23–30.
94. Martin P, Dabert M, Dabert J. Molecular evidence for species separation in the water mite *Hygrobates nigromaculatus* Lebert, 1879 (Acari, Hydrachnidia): evolutionary consequences of the loss of larval parasitism. Aquat Sci. 2010;72:347–60.
95. Collins RA, Cruickshank RH. The seven deadly sins of DNA barcoding. Mol Ecol Resour. 2013;13:969–75.
96. Zhang AB, Muster C, Liang HB, Zhu CD, Crozier R, Wan P. A fuzzy-set-theory-based approach to analyse species membership in DNA barcoding. Mol Ecol. 2012;21:1848–63.
97. García-Jiménez R, Horreo JL, Valdecasas AG. Minimal barcode distance between two water mite species from Madeira Island: a cautionary tale. Exp Appl Acarol. 2017;72:133–43.
98. Yu G, Rao D, Matsui M, Yang J. Coalescent-based delimitation outperforms distance-based methods for delineating less divergent species: the case of *Kurixalus odontotarsus* species group. Sci Rep. 2017;7:16124.
99. Liu XF, Yang CH, Han HL, Ward RD, Zhang AB. Identifying species of moths (Lepidoptera) from Baihua Mountain, Beijing, China, using DNA barcodes. Ecol Evol. 2014;4:2472–87.
100. Klimov PB, Skoracki M, Bochkov AV. *Cox*1 barcoding *versus* multilocus species delimitation: validation of two mite species with contrasting effective population sizes. Parasit Vectors. 2019;12:8.
101. Weigand H, Weiss M, Cai H, Li Y, Yu L, Zhang C. Deciphering the origin of mito-nuclear discordance in two sibling caddisfly species. Mol Ecol. 2017;5:705–15.
102. Toews DPL, Brelsford A. The biogeography of mitochondrial and nuclear discordance in animals. Mol Ecol. 2012;21:3907–30.
103. Edwards S, Bensch S. Looking forwards or looking backwards in avian phylogeography? A comment on Zink and Barrowclough 2008. Mol Ecol. 2009;18:2930–3.
104. Zink RM, Barrowclough GF. Mitochondrial DNA under siege in avian phylogeography. Mol Ecol. 2008;17:2107–21.
105. Arabi J, Judson MLI, Deharveng L, Lourenço WR, Cruaud C, Hassanin A. Nucleotide composition of CO1 sequences in Chelicerata (Arthropoda): detecting new mitogenomic rearrangements. J Mol Evol. 2012;74:81–95.
106. Elbrecht V, Leese F. Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. Front Environ Sci. 2017;5:11.
107. Leray M, Yang JY, Meyer CP, Mills SC, Agudelo N, Ranwez V. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Front Zool. 2013;10:34.
108. Deagle BE, Jarman SN, Coissac E, Pompanon F, Taberlet P. DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biol Lett. 2014;10:2–5.
109. Vamos E, Elbrecht V, Leese F. Short COI markers for freshwater macroinvertebrate metabarcoding. Metabarcod Metagenom. 2017;1:e14625.
110. Brandon-Mong G-J, Gan H-M, Sing K-W, Lee P-S, Lim P-E, Wilson J-J. DNA metabarcoding of insects and allies: an evaluation of primers and pipelines. Bull Entomol Res. 2015;105:717–27.
111. Zhang GK, Chain FJJ, Abbott CL, Cristescu ME. Metabarcoding using multiplexed markers increases species detection in complex zooplankton communities. Evol Appl. 2018;11:1901–14.
112. Elbrecht V, Taberlet P, Dejean T, Valentini A, Usseglio-Polatera P, Beisel J-N. Testing the potential of a ribosomal 16S marker for DNA metabarcoding of insects. PeerJ. 2016;4:e1966.
113. Więcek M, Martin P, Lipinski A. Water mites as potential long-term bioindicators in formerly drained and rewetted raised bogs. Ecol Indic. 2013;34:332–5.
114. Goldschmidt T. Water mites (Acari, Hydrachnidia): powerful but widely neglected bioindicators—a review. Neotrop Biodivers. 2016;2:12–25.
115. Miccoli FP, Lombardo P, Cicolani B. Indicator value of lotic water mites (Acari: Hydrachnidia) and their use in macroinvertebrate-based indices for water quality assessment purposes. Knowl Manag Aquat Ecosyst. 2013;411:08.
116. Pešić V, Smit H. Evidence of cryptic and pseudocryptic speciation in *Brachypodopsis baumi* species complex (Acari, Hydrachnidia, Aturidae) from Borneo, with description of three new species. Syst Appl Acarol. 2016;21:1092–106.
117. Pešić V, Smit H. *Neumania kyrgyzica* sp. nov. a new water mite from Kyrgyzstan based on morphological and molecular data (Acari, Hydrachnidia: Unionicolidae). Syst Appl Acarol. 2017;22:885–94.
118. Wiecek M, Martin P, Gabka M. Distribution patterns and environmental correlates of water mites (Hydrachnidia, Acari) in peatland microhabitats. Exp Appl Acarol. 2013;61:147–60.

Publisher's Note

Chapter III

Intra-Alpine Islands: Population genomic inference reveals high degree of isolation between freshwater spring habitats

Published in *Diversity & Distributions* (2021)

Published article on pages: 39 – 53

The article and accompanying supplementary information are available at:

https://doi.org/10.1111/ddi.13461

The water mite species *Partnunia steinmanni* of the Val Ftur spring in the Swiss National Park.

Received: 22 August 2021 | Revised: 18 November 2021 | Accepted: 24 November 2021

DOI: 10.1111/ddi.13461

RESEARCH ARTICLE

Diversity and Distributions WILEY

Intra-Alpine Islands: Population genomic inference reveals high degree of isolation between freshwater spring habitats

Lucas Blattner[1] | Kay Lucek[2] | Nathanael Beck[1] | Daniel Berner[3] | Stefanie von Fumetti[1]

[1]Department of Environmental Sciences, Geoecology, University of Basel, Basel, Switzerland

[2]Department of Environmental Sciences, Plant Ecology and Evolution, University of Basel, Basel, Switzerland

[3]Department of Environmental Sciences, Animal Diversity and Evolution, University of Basel, Basel, Switzerland

Correspondence
Lucas Blattner, Department of Environmental Sciences, Geoecology, University of Basel, Klingelbergstrasse 27, 4056 Basel, Switzerland.
Email: lucas.blattner@unibas.ch

Funding information
Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Grant/Award Number: 31003A_176234

Editor: Chris Burridge

Abstract

Aim: Alpine spring ecosystems have long been considered as highly isolated, island-like habitats. This presumption, however, is insufficiently supported empirically and conclusions about spring isolation have been based on indirect evidence. Therefore, we investigated the population genomic structure of *Partnunia steinmanni* Walter, 1906, a strictly spring-dwelling water mite (Hydrachnidia) species, to shed light on the degree of interconnection among freshwater spring habitats.

Location: Protected areas across the Alps, Central Europe.

Methods: *Partnunia steinmanni* populations were sampled by hand-net from 12 springs. Population genomic structure was inferred with 2263 polymorphic restriction site-associated DNA (RADseq) loci of 256 individuals. We assessed genomic admixture, the phylogenetic relationship, isolation by distance, contemporary migration, effective population sizes, and genetic diversity among individuals from different springs.

Results: We observed strong genetic differentiation between individuals from different springs. Water mites from each spring qualified as well-delimited distinct populations with only little intra-spring migration, even when these were located in close geographic proximity. Furthermore, we found subtle shared genetic structure among springs within the same area, and a southwestern genotype associated with the Rhône catchment that extended into eastern populations. Effective population size estimates and standing genetic variation within springs were generally low.

Main conclusions: Our findings indicate strong insularity of freshwater springs and headwater areas, likely caused by intra-alpine Pleistocene isolation and limited dispersal abilities of strictly spring-bound species like *P. steinmanni*. Our results support the concept of spring habitat isolation and highlight the importance of alpine protected areas to conserve springs as substantial components of freshwater biodiversity.

KEYWORDS
Alps, freshwater springs, hydrachnidia, insular-habitats, population structure, RADseq

1 | INTRODUCTION

Headwater stream sections and particularly springs, so-called crenic habitats, feature distinct environmental conditions and are inhabited by characteristic species assemblages (Cantonati et al., 2020; Di Sabatino et al., 2021; Glazier, 2014). As interface ecosystems between groundwater aquifer and surface streams (Manenti & Piazza, 2021; Stevens et al., 2021), springs show highly heterogenous microhabitat structures and steep environmental gradients on a small spatial scale (Di Sabatino et al., 2021; Reiss et al., 2016; Spitale et al., 2012). Species richness in springs is fostered by this high biological niche availability and is, among other taxa, extensively described for Hydrachnidia (Blattner et al., 2019; Gerecke et al., 2018; Pozojević et al., 2020), Diptera (Lencioni, Marziali, & Rossaro, 2011, 2012), Ostracoda (Rosati et al., 2017), diatoms (Cantonati et al., 2012; Lai et al., 2020; Pascual et al., 2020), and fungi (Wurzbacher et al., 2020).

Being biodiversity hotspots (Cantonati et al., 2012; Cartwright et al., 2020) and given their vulnerability to changing environmental conditions and human impact (e.g. Levison et al., 2014; Nielson et al., 2019; Stevens et al., 2021; Woodward et al., 2010), crenic habitats have increasingly become a focus for conservation research (Cantonati et al., 2021) aiming at decelerating ongoing biodiversity loss (Eisenhauer et al., 2019). Particularly alpine drainage basins and associated freshwater habitats gain attention because of their environmental sensitivity (e.g. Beniston, 2006; Gobiet & Kotlarski, 2020; Rogora et al., 2018) and function as potential refugia for threatened species (Cartwright et al., 2020). Efforts to monitor headwater environments and springs, aiming at discovering changing environmental integrity, are therefore continuously increased (e.g. Blattner et al., 2021; Cantonati et al., 2021; Küry et al., 2016).

Alpine springs and their associated biodiversity may particularly be vulnerable, when considered as isolated, island-like systems, surrounded by a terrestrial matrix that is presumably impermeable for aquatic organisms (Cantonati, Füreder, et al., 2012; Cartwright, 2019; Fattorini et al., 2016; Glazier, 2014; Von Fumetti & Blattner, 2017). This insularity putatively limits gene flow between populations, analogous to species on oceanic or sky islands (Rader et al., 2019; Sekar & Karanth, 2013), an idea introduced as the *isolated tributaries hypothesis* in alpine headwater streams (Ward, 1994). In the sense of classic island biogeography theory (MacArthur & Wilson, 1967), increased habitat isolation is predicted to negatively impact immigration, and accordingly the re-colonization rate, thus fostering genetic bottlenecks (Broquet et al., 2010; Pinheiro et al., 2017). Consequently, reduction of populations' resilience through diminished abilities to adapt to changing environmental conditions becomes likely (Sgrò et al., 2011). Decreasing population size due to anthropogenic impact may further exacerbate this effect (Elsen & Tingley, 2015; Shama et al., 2011).

However, conclusions about the degree of isolation of spring habitats have mainly been based on assumptions derived from community composition changes within small-scale study areas and thus are primarily indirect evidence for the interconnection between habitat patches (e.g. Fattorini et al., 2016; Von Fumetti & Blattner, 2017). Studies explicitly investigating spring population interconnection based on genetic structure are still scarce, limited in marker resolution to few traditional genetic loci, and/or conducted in extreme environments such as deserts dominated by endorheic and subsurface basins that are difficult to compare to other environments (e.g. Adams et al., 2018; Myers et al., 2001; Stutz et al., 2010).

FIGURE 1 Living *Partnunia steinmanni* specimen

To empirically assess alpine crenic habitat interconnection, we investigated the crenobiontic water mite species *Partnunia steinmanni* Walter, 1906 (Figure 1) that exhibits a primarily alpine distribution area with additional records from the Tatra and Western European lower mountain ranges (Gerecke, 1993). The genus *Partnunia* Piersig, 1896 includes in total ten species described from Europe and Asia (Gerecke, 1996) and belongs to the Hydryphantoidea, a phylogenetically basal water mite superfamily that was recently recognized as monophylum (Blattner et al., 2019; Dabert et al., 2016; Di Sabatino et al., 2010). In addition to *P. steinmanni*, only the species *P. angusta* (Koenike, 1893) has been described in Central Europe and assigned to the genus *Partnunia*, which exhibits relatively homogenous morphology (Di Sabatino et al., 2010; Gerecke, 1993). *Partnunia angusta* is restricted to the Alps and northern Prealps and in addition to springs also appears in spring brooks and low order streams (Di Sabatino et al., 2010). We focused on the strictly crenobiontic *P. steinmanni* that shows strong habitat preference for shaded springs dominated by moss and gravel substrate (Gerecke, 1993). *Partnunia steinmanni* were sampled in 12 springs located in different major protected areas across the Alps, and restriction site-associated DNA

TABLE 1 Locations of sampled springs

Spring	Protected area	Catchment	Location	Country	Latitude [WGS84]	Longitude	Elevation [m a.s.l.]	Sampling
MOL	Mercantour National Park	Rhône	Mollières	France	44.148663	7.190604	1671	09.08.19
RIF	Rifelbord Wildlife Area	Rhône	Zermatt	Switzerland	46.004263	7.739311	1781	17.10.20
VAG	Adamello-Brenta Nature Park	Po	Val d'Agola	Italy	46.173078	10.826485	1522	08.10.20
BRE	Adamello-Brenta Nature Park	Po	Val Brenta	Italy	46.187843	10.851127	1436	07.10.20
RIS	Adamello-Brenta Nature Park	Po	Tovel	Italy	46.250813	10.953048	1592	06.10.20
VA4	Swiss National Park	Danube	Plan da l'Acqua Suot	Switzerland	46.639530	10.176970	1710	10.10.20
VF3	Swiss National Park	Danube	Val Ftur	Switzerland	46.670605	10.194943	1970	11.10.20
VS6	Swiss National Park	Danube	Val da Stabelchod	Switzerland	46.667029	10.246264	2075	11.10.20
HIB	Berchtesgaden National Park	Danube	Hirschbichl	Germany	47.557212	12.803516	1268	01.10.20
KOB	Gesäuse National Park	Danube	Johnsbach Koderalm	Austria	47.545880	14.631686	1290	04.10.20
KOE	Gesäuse National Park	Danube	Johnsbach Kölbelalm	Austria	47.533333	14.643078	1117	04.10.20
GSC	Gesäuse National Park	Danube	Johnsbach Neuburgalm	Austria	47.521205	14.679822	1540	03.10.20

sequencing (RADseq) was performed. Subsequently, we inferred intra- and inter-population genomic structure, determined the influence of spatial structure on the genetic differentiation, and calculated demographic estimates to assess the degree of spring habitat isolation and estimate the genetic diversity of a characteristic crenobiontic species.

2 | METHODS

2.1 | Study sites, sampling and pre-processing

Partnunia steinmanni specimens (Figure 1) were sampled with a hand net (100 μm) in springs located in six main protected areas across the Alps in summer 2019 and 2020 (Table 1). The northernmost site was sampled in the Berchtesgaden National Park (Berchtesgaden NP) in Germany and the southernmost was located in the Mercantour National Park (Mercantour NP) in southeastern France. Three populations were sampled in the Adamello-Brenta Nature Park in the Trentino region of northern Italy (Adamello-Brenta NP), and the *locus typicus* population of *P. steinmanni* was collected in the Rifelbord Wildlife Area (Rifelbord WA) near Zermatt in Switzerland. Additionally, we included three populations located in the highly protected Swiss National Park (Swiss NP) (IUCN category 1a), which belongs to the central part of the Alps, and three populations in the Austrian Gesäuse National Park (Gesäuse NP), representing the easternmost sampling area. The different protected areas constitute different main drainage basins across the Alps, including the Rhône (Mercantour NP and Rifelbord WA), Po (Adamello-Brenta NP), and Danube (Swiss NP, Berchtesgaden NP, and Gesäuse NP) catchments (see e.g. Winterberg & Willett, 2019) (Table 1). Sampling permissions were granted for each protected area by the respective authorities.

Each spring population consisted of ≥30 individuals sorted out alive directly in the field, and each individual was transferred to a single well of a 48-well cell culture plate (Sarstedt AG & Co. KG, Nümbrecht, Germany) prefilled with water directly from the spring. The plates were held at 4°C for one week until the specimens were subsequently transferred to molecular grade ethanol (100%) and stored at −20°C until further processing. This procedure resulted in a starvation period with the aim to reduce possible sample contamination due to residual gut content, which potentially can be detected until one week after starvation (Martin et al., 2015).

2.2 | DNA extraction, RADseq library preparation and sequencing

Each mite was first submerged in molecular grade water to remove residual ethanol. DNA of 25 individuals per site was extracted and purified by applying the SPRI bead-based DNAdvance Kit (Beckman Coulter Live Sciences, Indianapolis, USA). Whole mite individuals were processed in 96 deep well plates (Eppendorf, Hamburg, Germany) according to manufacturer protocol with a final elution

volume of 100 µl. The resulting DNA eluates were quantified with the Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, USA). 21–22 individuals per population, showing the highest amount of extracted DNA, were chosen to be further processed, resulting in a total of 256 *P. steinmanni* specimens (Appendix S1).

Due to relatively low initial DNA yield (mean ± SD: 0.2 ± 0.15 ng/µl, Appendix S1) that was obtained from the water mites, a multiple displacement amplification (MDA) was performed for each individual to increase the amount of DNA. This procedure has proven to work well for RADseq, without introducing notable genotyping bias as shown in previous studies (Blair et al., 2015; Cruaud et al., 2018; De Medeiros & Farrell, 2018). For MDA, the REPLI-g Mini kit (Qiagen, Hilden, Germany) was applied to each sample with 5 µl of DNA eluate resulting in 161 ± 23.7 ng (mean ± SD, Appendix S1) amplified genomic DNA.

RADseq libraries were prepared following Ali et al., 2016, using the same 8 bp Hamming-distance optimized barcodes from Kozarewa & Turner, 2011. Each whole genome amplified DNA extract was standardized to 300 ng DNA and subsequently digested with 10 units of PstI restriction enzyme (New England Biolabs Inc., NEB, Ipswich, USA), 1X NEB buffer 3.1, and molecular grade H_2O for 60 min at 37°C and inactivated at 80°C for 20 min. The double-stranded barcode adapters were then directly sticky-end ligated to the restriction digested DNA with 1 µl T4 DNA Ligase [400,000 units/ml] (NEB) and 1× T4 buffer (NEB) at 16°C overnight with subsequent heat inactivation for 10 min at 65°C. After barcode ligation, 125 ng of DNA from each sample were pooled to 8 sequencing libraries (named Ps1–Ps8), containing 32 samples and 4 µg digested and adaptor-ligated DNA fragments each (see Appendix S1 for detailed library affiliation of individuals). After pooling, libraries were purified and concentrated with a 1.8X SPRIselect (Beckman Coulter) bead clean-up and eluted in 200 µl TE low EDTA buffer (10 mM TRIS-HCL, 0.1 mM EDTA, pH 8).

Cleaned RADseq libraries were then sheared on a Bioruptor NGS (Diagenode SA, Seraing, Belgium) to a final 200–500 bp insert size at 4°C with 3 × 3 cycles, each consisting of a 30-s ON and 60-s OFF period. Appropriate size distribution was evaluated on a Bioanalyzer 2100 system (Agilent Technologies Inc., Santa Clara, USA) using the high sensitivity DNA kit.

After shearing, RAD fragments containing the biotinylated barcode adapters, were physically isolated with Dynabeads™ M-280 Streptavidin beads (Thermo Fisher Scientific) according to manufacturer protocol with a final resuspension in 40 µl of 1× NEB buffer 4 (NEB). Due to the SbfI restriction site containing adaptor sequences, the RAD fragments were then separated from the streptavidin beads by performing a SbfI (NEB) digestion at 37°C for 60 min, followed by a 1.8× SPRIselect (Beckman Coulter) bead clean-up on the supernatant with a final elution in 50 µl of TE low EDTA buffer.

To finalize the RADseq library preparation and incorporate Illumina® compatible sequencing adapters, the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (NEB) in combination with the NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) (NEB) were applied according to the manufacturer protocol. This resulted in the final libraries having 57.2–77.2 ng/µl DNA, with each library being tagged by a unique primer combination. Fragment length distributions of the final libraries were assessed on a Bioanalyzer (Agilent).

Sequencing was subsequently performed by equimolarly pooling all 8 libraries together and applying the pool on 8 lanes of a HiSeq 4000 System with 150 paired-end cycles (Illumina Inc., San Diego, USA) by the Genomics Technologies Facility (GTF) (Lausanne, Switzerland). Nucleotide diversity at the first sequenced bases was increased by adding 1% bacteriophage PhiX genome (Illumina) in combination with 9% of a *P. steinmanni* WGS sample per sequencing lane. Short read data were uploaded to the European Nucleotide Archive (ENA) and are available under the study accession number: PRJEB47010.

2.3 | Raw data preparation, assembly and SNP calling

A total of 4.5×10^9 reads (approximately 5.6×10^8 reads per RADseq library) were obtained. Quality control of the raw data was performed with FastQC V0.11.8 (Andrews, 2010) and MultiQC V1.11 (Ewels et al., 2016). Remaining NEBNext adaptor sequences were trimmed from the 3' ends of the reads with cutadapt V3.4 (Martin, 2011), and all reads were standardized to 100 bp equal length.

To obtain individual-specific datasets, demultiplexing and quality filtering of the raw data was done with *process_radtags*, a component of the Stacks 2 V2.59 pipeline (Rochette et al., 2019), allowing one mismatch position in the barcode sequence (Appendix S1) and requiring an intact PstI restriction residual. Due to the use of blunt-end ligated sequencing adaptors, resulting in mixed orientation of forward and reverse reads, the *bestrad* option was enabled in *process_radtags* that automatically scans for the barcode sequence on either read and corrects the orientation.

Following Lucek et al., 2020, *ustacks* V2.59 (Rochette et al., 2019) was used to *de novo* assemble the pre-processed restriction site flanking forward reads with a minimum stack size of 50 reads, three allowed mismatches between reads to be associated with the same stack, disabled gapped-alignment and disabled haplotype calling from secondary reads. Reverse reads were not included because of high read overlap due to relatively small final insert sizes (± 200 bp) and therefore sequence redundancy. To account for putatively high interpopulation divergence caused by the large geographic extent of the study, the assembly was performed separately for individuals belonging to a specific sampling area resulting in six different assemblies. Homologous RAD loci were then identified between the different assemblies using *swarm* V3.1 (Mahé et al., 2021), merging contigs with 97% sequence similarity. Only contigs that occurred in all six initial assemblies were retained.

Resulting contigs were subsequently filtered for contaminants by blasting each contig against the NCBI GenBank nucleotide database (Agarwala et al., 2018, accessed: 15.3.2021) with the *blastn* function as implemented in BLAST+ V2.2.23 (Camacho et al., 2009).

This yielded 1516 final contigs that were not identified as derived from extraneous origin (e.g. bacteria, archaea or viruses).

The pre-processed raw reads were then aligned against the de novo assembly using minimap2 V2.17 (Li, 2018) in short read mode, and the resulting alignments were converted, sorted, and indexed with SAMtools V1.12 (Danecek et al., 2021). Next, the *mpileup* und *call* functions of BCFtools V1.10.2 (Danecek et al., 2021) were used with a maximum read depth filter of 5000 to call a total of 7069 SNPs. This preliminary genotype dataset was then filtered with VCFtools V0.1.17 (Danecek et al., 2011). Sites with more than 60% missing data, minor allele frequency of <0.01 and minor allele count of <3, genotype quality of <Q20, and read depth of <5× and >800× at the individual level were removed, as well as indel positions. Furthermore, 18 individuals showing >56% missing data were removed. This approach resulted in a final dataset of 2263 SNPs for 238 individuals with a mean read depth of 172× that was used for all subsequent genomic analysis.

2.4 | Population genomic and phylogenetic inferences

Physical variant linkage pruning was conducted in PLINK V1.90b6.24 (Chang et al., 2015) with a sliding window size of 50 variants, a step size to shift the window of 10 variants, and a r^2 threshold of .1 to obtain a SNP dataset with a subset of markers that are in approximate linkage equilibrium. Linkage pruning resulted in 1409 SNPs that were used to infer population structure with Admixture V1.3 (Alexander et al., 2009). Admixture was run assuming between 1 and 20 putative populations (*K*) and the most probable K was assessed by the Admixture cross-validation procedure. Additionally, a principal component analysis (PCA) was calculated in PLINK for the linkage pruned markers. Both analyses were post-processed and plotted in R V4.1.0 (R Core Team, 2021).

The relationship among all individuals was assessed with RAxML V8.2.12 (Stamatakis, 2014) under a generalized time-reversible (GTR) model of evolution with optimized substitution rates and gamma model of rate heterogeneity on the dataset without linkage pruning. To avoid disconcerting influence of admixed individuals on the general tree topology (Seehausen, 2004; Shirk et al., 2021), individuals showing >20% admixture were excluded. Because only polymorphic sites were used, a Lewis ascertainment bias correction (ASC_GTRGAMMA function in RAxML) was implemented, and statistical branch support was evaluated by computing 5000 bootstrap replicates. The resulting unrooted bipartition tree was visualized in TreeViewer V1.2.2 (Bianchini, 2021).

Isolation by distance (IBD) was assessed by first computing geographic distances in km between the sampling location as implemented in the geodist V0.0.7 (Padgham & Sumner, 2021) R package (Appendix S2). GenoDive V3.05 (Meirmans, 2020) was then used to calculate pairwise F_{ST} values between all combinations of *P. steinmanni* spring populations (Appendix S3), followed by a Mantel test (Mantel, 1967) with 20,000 permutations between log transformed geographic and genetic distance matrices. To further quantify the amount of genetic variance explained by the spatial structure, we performed a distance-based redundancy analysis (dbRDA) as implemented in the vegan V2.5-7 R package (Oksanen et al., 2020) with the genetic distances (pairwise F_{ST}) between populations as dependent variable. We calculated Moran Eigenvector Maps (MEMs) based on the geographic distances between the springs following Borcard et al. (2018) and Gibson and Moyle (2020) and processed them with the adespatial V0.3-14 (Dray et al., 2021) R package. Main catchment areas (Table 1) and MEMs were then included in the dbRDA as explanatory variables to represent spatial structure.

Furthermore, an analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was calculated with 10,000 permutations based on pairwise F_{ST} in GenoDive to assess the level of population differentiation. We tested for significant genetic differentiation among springs within, as well as between different sampling areas.

2.5 | Genetic diversity and demographic estimates

Genetic diversity of the different spring populations, expressed as expected (H_e) and observed (H_o) heterozygosity, as well as inbreeding coefficients (G_{IS}) were calculated in GenoDive. We estimated contemporary effective population size (N_e) of *P. steinmanni* with a bias-corrected version of the linkage disequilibrium method (Waples & Do, 2008), by using NeEstimator V 2.1 (Do et al., 2014), assuming random mating and comparing allele frequencies of ≤0.05, ≤0.02 and ≤0.01.

Lastly, recent migration rates between springs were assessed by implementing BA3-SNPs V 3.0.4 (Mussmann et al., 2019), a modification of BayesAss (Wilson & Rannala, 2003) that allows handling of large SNP datasets. First, we assessed the optimal mixing parameters for migration rates (deltaM = 0.1), allele frequencies (delta = 0.55), and inbreeding coefficients (deltaF = 0.0375) by running ten repetitions in BA3-SNP-autotune V 3.0.4 as recommended by Mussmann et al. (2019). Subsequently, BA3-SNPs was run with the predefined mixing parameters for 50 million generations, sampling every 100th generation. The first million generations were discarded as burn-in and chain convergence was assessed in Tracer V 1.7.1 (Rambaut et al., 2018).

3 | RESULTS

3.1 | Population genomic structure

The best-supported number of genetic clusters (i.e. lowest standard error of cross-validation error estimate) identified by Admixture was 12 (Appendix S4), corresponding precisely to the number of sampled springs (Figures 2 and 3a). Overall, each spring thus consisted of individuals exhibiting a spring-specific genotype. Furthermore, admixture tended to be slightly more pronounced between springs within sampling areas, for example between the VA4 and VF3 populations located in the Swiss NP area or between GSC and KOE in the Gesäuse NP (Figure 2).

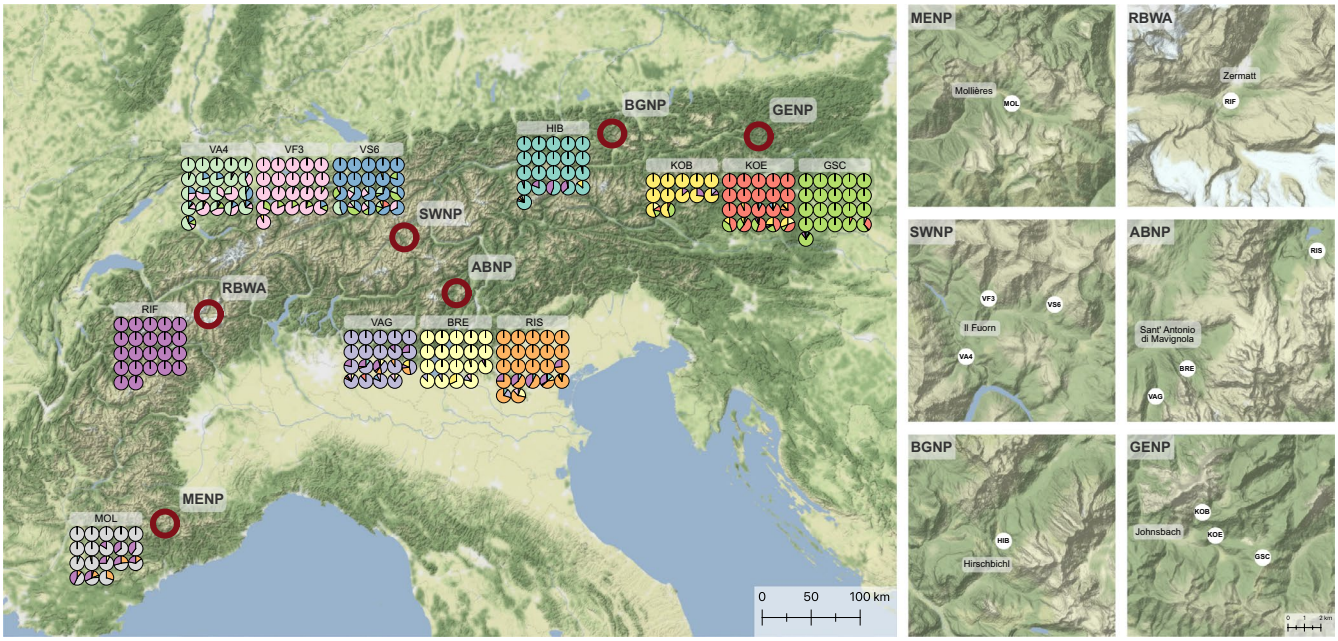


FIGURE 2 Genetic structure between sampling areas and location of *P. steinmanni* populations. The map of the whole sampling area (left) shows admixture population assignments as pie diagram for each individual, arranged according to springs and displayed adjacent to the respective sampling area, indicated as red circles. Areas are abbreviated as MENP (Mercantour NP), RBWA (Rifelbord WA), SWNP (Swiss NP), ABNP (Adamello-Brenta NP), BGNP (Berchtesgaden NP) and GENP (Gesäuse NP). Each colour represents a distinct group assignment at $K = 12$. Abbreviations and colours are identical to Figure 3. The excerpts on the right side show the exact locations of the springs within each of the six sampling areas. The maps were created in QGIS V3.18 with base map tiles by Stamen Design (http://maps.stamen.com), under CC BY 3.0. Data by OpenStreetMap, under ODbL

Interestingly, however, the genomic background characteristic for the *locus typicus* population (RIF) also appeared with differing extent in several other, sometimes quite distant regions and spring populations, including in the MOL (Mercantour NP); KOB, KOE and GSC (Gesäuse NP); and HIB (Berchtesgaden NP) springs (Figure 3a).

The same applies to VAG and RIS springs in the Adamello-Brenta NP (Figure 2). However, BRE, which is in close proximity to VAG, shows only one individuum with minor assignment to the RIF genotype. Furthermore, the RIF genotype is completely absent in springs from the Swiss NP area (Figure 3a).

The sampled *P. steinmanni* populations in the Swiss National Park (VA4, VF3 and VA6), as well as the populations located in the Gesäuse NP area, show relatively high proportions of individuals with shared genetic structure between springs (Figures 2 and 3a).

The two leading PCA axes together explained 30.4% of the total variance, and individuals from different areas were often separated (Figure 3b and Appendix S5). PC2 mainly separated individuals belonging to the western populations located in the Mercantour NP and Rifelbord WA sampling areas from all other, more northeastern individuals. Individuals belonging to the other springs were grouped in sampling area-specific clusters by PC1 (Figure 3b).

In line with the admixture and principal component analysis, the RAxML inference revealed distinct clades, primarily separating individuals by springs (Figure 3c). Here, the sampling areas Mercantour NP and Rifelbord WA clustered more closely together, showing increased relatedness between these westernmost populations.

In contrast to the admixed individuals (Figure 3a), individuals from the Adamello-Brenta NP show clade association congruent with the geographic proximity of springs. Likewise, individuals from the Swiss NP as well as Gesäuse NP springs branch by geographic proximity (Figures 2 and 3c). Furthermore, the sprigs located in the central Alps, Swiss- and Adamello-Brenta NP, seem to be closer related to each other than to the peripheral sites, although statistical bootstrap support was weak.

3.2 | Population differentiation and isolation by distance

The analysis of molecular variance (AMOVA) revealed the strongest genetic differentiation among the sampling areas, that is, among national parks (29% of observed variation, $F_{ST} \pm SD = 0.34 \pm 0.005$, $p < .001$). A significant fraction of the total variation, however, was also explained by the springs within the areas (21% of observed variation, $F_{ST} \pm SD = 0.21 \pm 0.008$, $p < .001$). Pairwise F_{ST} between the geographically closest spring populations within the Gesäuse NP, Swiss NP and the Adamello-Brenta NP was consistently higher than 0.18 (Appendix S3). In contrast, genetic variation among individuals within springs was very low (0.8% of observed variation, $F_{ST} \pm SD = 0.01 \pm 0.009$, $p < .001$). Moreover, we found strong isolation by distance among the spring populations (Mantel test: $r = .66$, $p < .001$), confirmed by the dbRDA analysis. The four constrained axis of the dbRDA

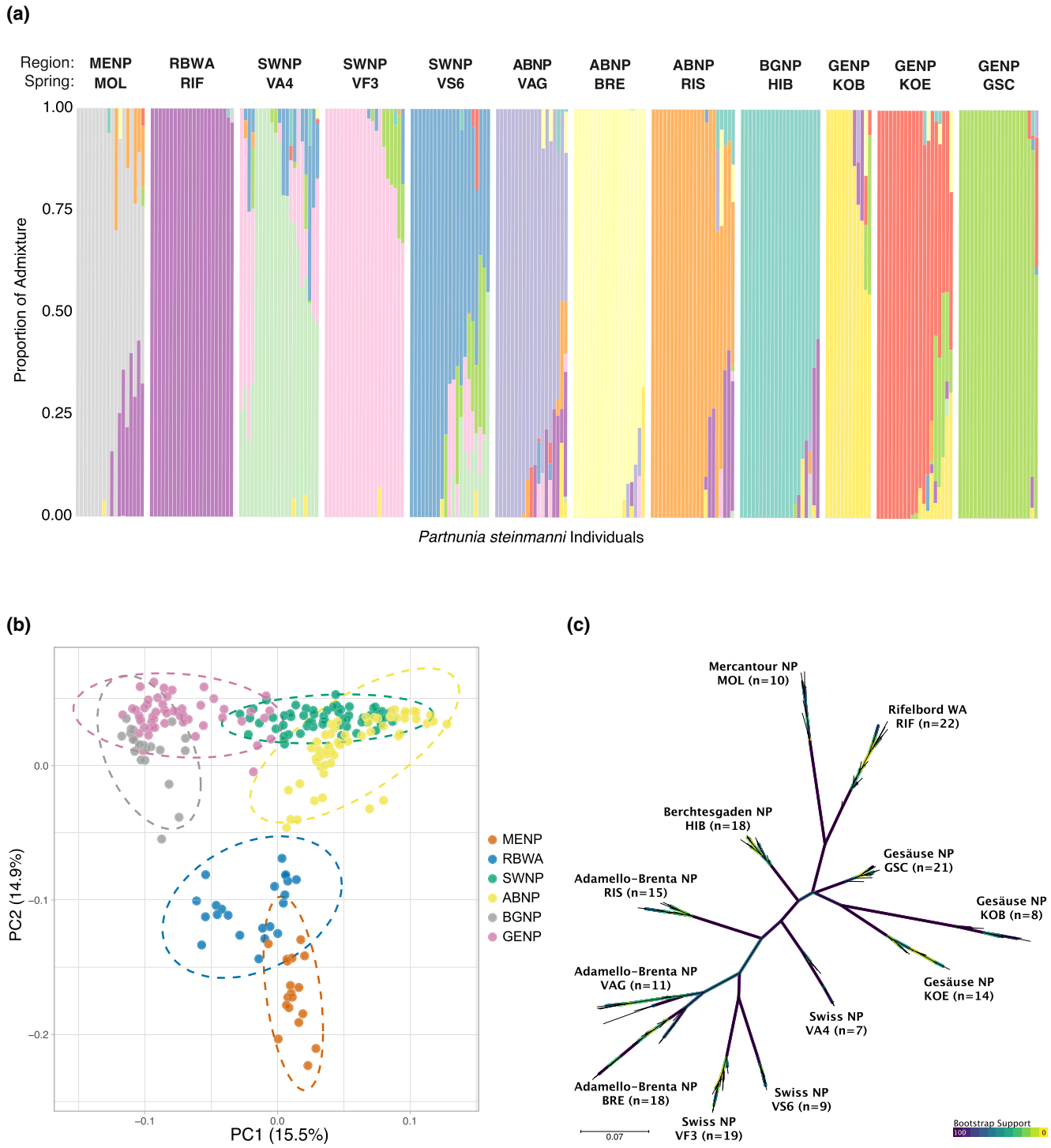


FIGURE 3 *Partnunia steinmanni* population differentiation. (a) Admixture analysis of the spring populations. The putative admixture population assignments of individuals belonging to different spring populations and areas, arranged from west to east, at $K = 12$ of the linkage pruned dataset. Each column represents the genetic background of one spring population. Colour coding and area abbreviations are identical to Figure 2. (b) Genetic principal component analysis highlighting the genetic structuring between sampling areas. Individuals and 95% confidence ellipses are coloured according to the different sampling areas, which are abbreviated as in (a) and Figure 2. (c) Unrooted maximum likelihood tree of *P. steinmanni* spring populations. Bootstrap support based on 5000 replicates is shown as colour-coded branches

explained 51.7% of the total genetic variance and the ordination revealed a distinct clustering of the genetic distances between springs by spatial structure, that is, their geographic proximity and catchment areas (Figure 4).

3.3 | Genetic diversity and demographic estimates

Overall, relatively low and homogenous levels of genetic diversity was observed between springs (mean $H_e \pm$ SD = 0.113 ± 0.023;

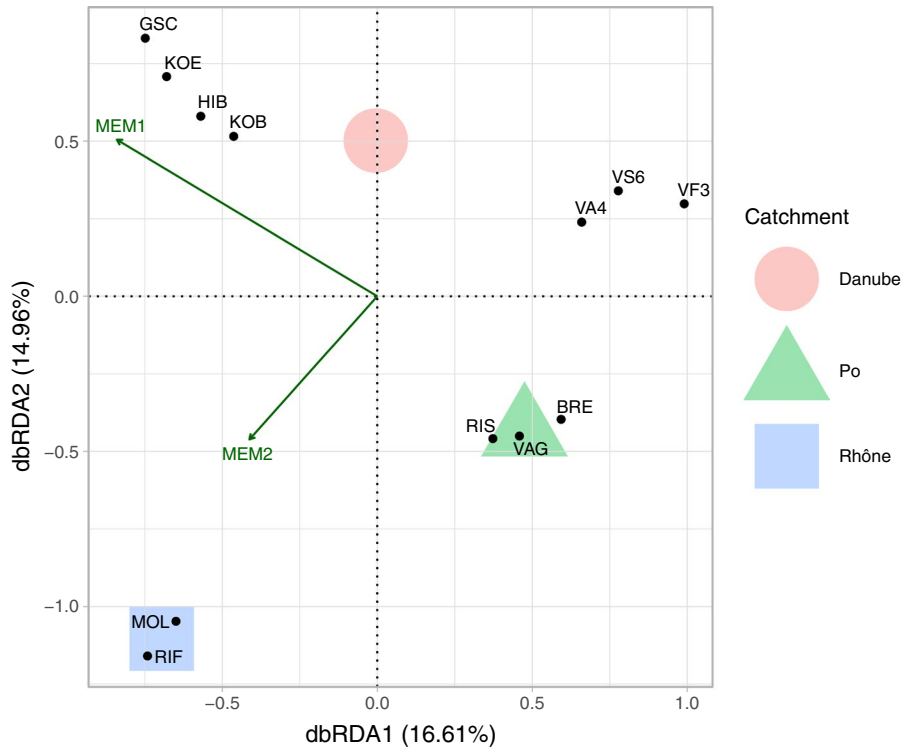


FIGURE 4 Distance-based redundancy analysis (dbRDA) of genetic distances and spatial structure. Pairwise F_{ST} were used as dependent variables and geographic distances (Appendix S2) as well as the main catchment areas across the Alps were included as explanatory variables to assess the influence of spatial structure on the *P. steinmanni* population differentiation. The first two plotted axis explain 31.3% of the variance and all four constrained axis 56.7%. The catchments (categorial variables) are plotted as centroids and geographic distances expressed as Moran Eigenvector Maps (MEMs) are shown as arrows

TABLE 2 Genetic diversity and effective population size estimates of *P. steinmanni* spring populations. For each spring, the sample size (N), the observed (H_o) and expected (H_e) heterozygosity and the level of inbreeding (GIS) are given. Average effective population sizes (N_e) with the respective 95% confidence intervals (CI) are moreover provided (see Appendix S7 for details). Due to infinite CI, Ne could not be estimated for population KOB

Spring	Protected Area	N	H_o	H_e	G_{IS}	N_e	[95% CI]
MOL	Mercantour National Park	18	0.134	0.139	0.038	10.7	[10.2; 11.1]
RIF	Rifelbord Wildlife Area	22	0.057	0.075	0.249	18.5	[15.3; 23.1]
VAG	Adamello-Brenta Nature Park	19	0.128	0.141	0.093	5.1	[4.8; 5.4]
BRE	Adamello-Brenta Nature Park	19	0.132	0.131	−0.006	11.6	[11.1; 12.0]
RIS	Adamello-Brenta Nature Park	22	0.088	0.100	0.125	6.2	[5.9; 6.5]
VA4	Swiss National Park	21	0.141	0.150	0.061	6.6	[6.4; 6.7]
VF3	Swiss National Park	21	0.102	0.098	−0.038	12.1	[11.7; 12.5]
VS6	Swiss National Park	21	0.122	0.125	0.021	6.8	[6.5; 7.2]
HIB	Berchtesgaden National Park	21	0.111	0.107	−0.037	6.4	[6.1; 6.7]
KOB	Gesäuse National Park	13	0.128	0.172	0.257	–	[–; –]
KOE	Gesäuse National Park	20	0.108	0.127	0.148	15.8	[14.3; 17.7]
GSC	Gesäuse National Park	21	0.105	0.096	−0.103	9.0	[8.6; 9.4]

mean H_o ± SD = 0.122 ± 0.0273) (Table 2). The RIF spring population showed the lowest expected and observed heterozygosity. Interestingly, RIF and KOB also exhibit increased levels of inbreeding compared to the other populations. BRE, VF3, HIB and GSC showed no evidence for putative inbreeding. Effective population size for *P. steinmanni* was low and on average estimated at 9.9 ± 4.3 (mean ± SD) individuals (Table 2 and Appendix S6). Due to infinite confidence intervals, KOB failed at providing reliable N_e estimates, likely caused by sampling bias due to the low amount of individuals processed (Do et al., 2014). The assessment of contemporary migration revealed very low migration rates (m) between (mean m ± SD = 0.01 ± 0.006) and genetic exchange almost exclusively

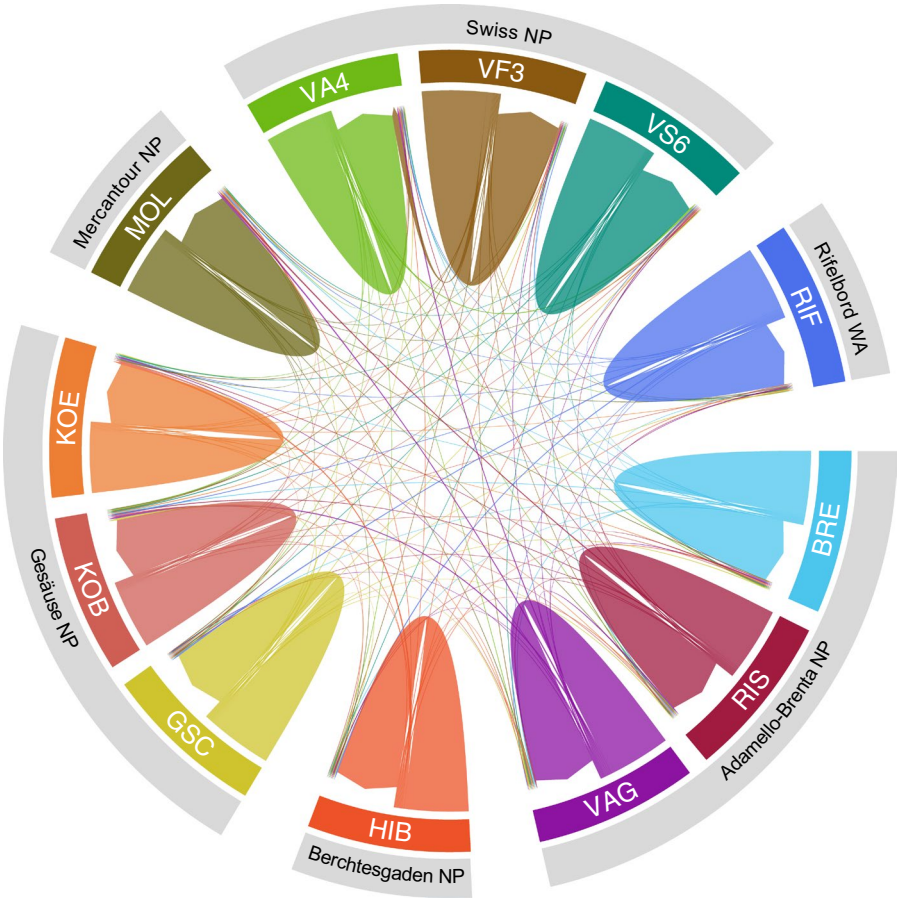


FIGURE 5 Contemporary migration rate estimates between spring populations. Directional migration was calculated by using BA3-SNP and is represented as arrows, whose sizes are relative to the proportion of migrating individuals and colour-coded according to their population of origin. Exact migration rates, and accordingly number of individuals relative to absolute population sizes can be seen in Appendix S7

within springs (mean m ± SD = 0.87 ± 0.02). Only from VF3 to VA4, both springs located in the Swiss NP, a slightly higher migration rate of approximately 6% (m = 0.06; 1.2 out of 21 individuals) occurred (Figure 5 and Appendix S7).

4 | DISCUSSION

Spring ecosystems have been considered as putatively isolated insular habitats despite limited empirical evidence (e.g. Cantonati et al., 2006; Fattorini et al., 2016). To evaluate this concept, we here investigated the population genetic structure of a strictly spring-dwelling water mite species within and among protected areas across the Alps. We show that even geographically close populations of *P. steinmanni* exhibit spring-specific genotypes, and vast genetic differentiation combined with limited inter-spring migration. This provides strong evidence of restricted dispersal and gene flow, consistent with an island-like habitat character of alpine spring ecosystems. Low degree of habitat interconnection between springs has already been inferred indirectly based on macroinvertebrate and spring-related stygofauna community composition data (Fattorini et al., 2016; Von Fumetti & Blattner, 2017), springs from extreme environments (Myers et al., 2001), or species that are not exclusively spring-bound (Engelhardt et al., 2011). Our study now offers population genomic support of the idea that alpine spring ecosystems represent island-like habitats.

Landscape-dependent population structure is, among other factors, influenced by species-specific dispersal abilities that determine the relevance of putative barriers, such as topographic and environmental gradients (Garant et al., 2007; Storfer et al., 2010; Van Buskirk & Jansen van Rensburg, 2020). Springs are known to harbour diverse species assemblages with different dispersal capacities (e.g. Stevens et al., 2021), including strongly or weakly dispersing taxa (De Bie et al., 2012). Consequently, depending on the study organism, the degree of geographic isolation may differ, potentially leading to opposing conclusions about spring interconnection.

In contrast to other spring-dwelling taxa, water mites such as *P. steinmanni* show live-stage-dependent dispersal abilities. As larva, they parasitize insect imagines with differing flight abilities, putatively allowing for effective dispersal, and are as adults restricted to a single spring (Martin & Stur, 2006; Zawal, 2003). The host species is, however, potentially impacted by parasite load, which may alter its flight capacities and consequently restricts its dispersal abilities and migration distances (Sánchez et al., 2015; Smith, 1988). Water mites

can thus be considered putatively poor dispersers, and the degree of habitat isolation in water mites can be expected stronger compared to efficiently dispersing crenobiontic species. However, clear estimates of the intensity of the impact of parasitism on the host species in spring ecosystems is not evident and needs further research. In contrast to the assumed diminished dispersal of hosts and depending on the respective host species, an unimpaired host dispersal could appear despite parasitism. Consequently, our results showing spring insularity putatively also apply to the host species, which in the case of *P. steinmanni* consist of at least three insect orders (Plecoptera, Trichoptera and Diptera) representing the majority of spring-bound, and even spring-related species (Martin et al., 2009).

The overall population structure suggests a southwestern lineage represented by the populations MOL and RIF that are associated with the Rhône catchment, with individuals sharing parts of this genomic background occurring mainly in the geographically close Adamello-Brenta NP area and to a limited degree in the eastern Alps (Figure 2). Similar biogeographic patterns have been observed in a tufa stream adapted Trichoptera species (Engelhardt et al., 2011) and were shown across the Alps for both, terrestrial and aquatic taxa, to be associated with post-glacial recolonization from distinct refugia (Asztalos et al., 2021; Hewitt, 2000; Lucek et al., 2020). In contrast to that, the Swiss National Park exhibits a unique genomic setting with only a single individual in the VS6 spring showing some genetic similarity with the KOE spring of the Gesäuse NP region. This may suggest that local topography could have acted as a barrier and hindered the western lineage putatively originating in the Rhône drainage basin from spreading directly northwards; however, denser sampling of possible contact zones would be necessary to support this assumption.

Apart from these general patterns, the overall high degree of genetic differentiation between *P. steinmanni* populations from different springs (Appendix S3—F_{ST}) may have originated as a result of many different intra-alpine glacial refugia possibly associated with main catchment areas, where the species was able to survive locally during glacial periods and experienced post-glacial isolation due to limited dispersal capacities, analogous to the alpine caddisfly species *Drusus discolor* (Rambur, 1842) (Pauls et al., 2006).

The observed genetic differentiation among springs could also reflect isolation caused by local adaptation to different microhabitats in springs. However, the influence of isolation by distance vs. isolation by environment on population structure is discussed controversially (e.g. Aguillon et al., 2017; Sexton et al., 2014; Van Buskirk & Jansen van Rensburg, 2020) and seems to be taxon, environment and time-scale-dependent. By assessing the correlation between geographic and genetic distance, we were able to show the presence of isolation by distance (IBD) among springs. *Partnunia steinmanni* is known to be strictly restricted to alpine crenic habitats and prioritizes a specific benthic microhabitat, that is, gravel-dominated substrate rich in moss in forest or shaded locations (Gerecke et al., 2005, 2009). These environmental conditions are shared among all our sampling sites. Combined with relatively high environmental stability in springs (e.g. Cartwright et al., 2020; Di Sabatino et al., 2021), divergent natural selection through environment seems unlikely to be a strong contributor for the observed population differentiation observed among *P. steinmanni* populations. However, the dbRDA suggested that up to 56.7% of the total genetic differentiation between populations can be directly explained by spatial structure. It has been shown that *P. steinmanni* has a rather broad host species spectrum with taxa belonging to at least three different insect orders (Plecoptera, Trichoptera and Diptera) (Martin et al., 2009). Isolation by population-specific host species preference could thus have further shaped the population structure of *P. steinmanni*. High degree of association with spring-specific host species assemblages could potentially lead to a restricted dispersal to springs at close proximity and consequently foster genetic isolation. The exceptionally low between-spring migration rates shown by our results strengthen this assumption. Indeed, a slightly higher migration rate occurred between VF3 and VA4, where there is a lack of topographic barriers such as mountain massifs between these two springs (Figure 2). However, to describe additional processes potentially causing the strong population distinctiveness and isolation of springs and assess the influence of IBE, further investigations and thorough evaluation of putative environmental differences between *P. steinmanni* habitats need to be conducted.

Effective population sizes (N_e) were generally low, that is around 10 individuals per population, which is comparable to other parasitic Acari (see e.g. Huber et al., 2019). Compared to the census population sizes that can easily exceed tens and even hundreds of *P. steinmanni* individuals (e.g. Gerecke et al., 2009; Kreiner et al., 2018), the estimated N_e is relatively low. This may indicate a reduction of effective population size due to low migration and high isolation over time, implying a bottleneck scenario with putatively increasing influence of genetic drift. Consequently, genetic diversity loss and the possibility of reduced fitness of the species may occur (see e.g. Broquet et al., 2010; Charlesworth, 2009; Hohenlohe et al., 2021). Due to re-colonization of the periglacial inhabitable areas, we assume that founder effects could also have induced the relatively low N_e and reduced genetic diversity within springs (see e.g. Montero-Pau et al., 2018; Peter & Slatkin, 2015). The effect of strong genetic differentiation among and low genetic diversity within populations particularly applies for species inhabiting formerly glaciated areas (Galbreath & Cook, 2004; Pečnerová et al., 2017) and has been shown for other water mite species (Bohonak, 1999). Suitable estimation of effective population size, however, can be influenced by sampling strategy (Do et al., 2014; Hare et al., 2011). Especially in species that show pronounced spatial structure and isolation, single sample strategies, as N_e estimation based on LD, should be validated by temporal methods including samples of multiple generations (Neel et al., 2013).

The RIF spring population near Zermatt showed exceptionally low genetic diversity, and a relatively high inbreeding coefficient compared to the other springs, that revealed rather homogenous heterozygosity estimates. We assume that the geographic location of Zermatt, surrounded by pronounced mountain massifs, and therefore, high degree of topographic isolation (see Figure 2) has

potentially driven this pattern. The comparably high N_e that was estimated for this population may result from the above-mentioned sampling bias and should be validated by increasing the sample size, that is number of individuals.

Taxonomically, the description of *P. steinmanni* has been based on few individuals from very different geographic locations, including the *locus typicus* in the Rifelbord Wildlife Area near Zermatt, but also far distant springs in eastern Austria and even outside the Alps (Gerecke, 1993). The morphological characters of the a priori morpho-species are considered to be rather variable; thus, the appropriateness of the species delimitation should be questioned (R. Gerecke, personal communication). Furthermore, intra-species clade separation has been shown for *P. steinmanni* by investigating traditional genetic species delimitation markers (Blattner et al., 2019). Given the strong population structure, a thorough re-evaluation of the *P. steinmanni* morphospecies should be conducted to assess the possibility of *P. steinmanni* being a species complex rather than a single, well-defined species.

To conclude, our results provide strong evidence for a high degree of insularity of alpine spring habitats, likely shaped by Pleistocene isolation in different intra-alpine refugia associated with main catchment areas as previously shown to be characteristic for alpine headwater environments. Crenobiontic species with limited dispersal abilities and low inter-population migration rates such as *P. steinmanni* show restricted gene flow, resulting in high genetic diversity and the potential for speciation across the Alps. Each spring and study area contains populations with a unique genetic make-up, and the loss of individual habitats will directly drive a decrease in overall genetic diversity. Our work thus highlights the importance of protected areas, such as National Parks that limit anthropogenic impact, as archives of genetic biodiversity.

ACKNOWLEDGEMENTS

Field sampling coordination and project approval were granted through the Swiss National Park, Gesäuse National Park, Berchtesgaden National Park, Mercantour National Park, and Adamello-Brenta Nature Park administrations. Furthermore, J. Ebner (University of Basel), A. Escher (University of Basel), M. Roesti (University of Bern), and R. Gerecke (University of Tübingen) contributed valuable support during the project. Helpful advice on sequencing strategy was provided by the Genomic Technologies Facility Team (University of Lausanne). Calculations were performed at sciCORE (http://scicore.unibas.ch/) scientific computing centre at the University of Basel on their HPC infrastructure. This work was funded by the Swiss National Science Foundation (SNSF) [grant number 31003A_176234].

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ddi.13461.

DATA AVAILABILITY STATEMENT

Short read data were uploaded to the European Nucleotide Archive (ENA) and are available under the study accession number: PRJEB47010.

ORCID

Lucas Blattner https://orcid.org/0000-0002-1331-3482

REFERENCES

Adams, N. E., Inoue, K., Seidel, R. A., Lang, B. K., & Berg, D. J. (2018). Isolation drives increased diversification rates in freshwater amphipods. *Molecular Phylogenetics and Evolution*, *127*, 746–757. https://doi.org/10.1016/j.ympev.2018.06.022

Agarwala, R., Barrett, T., Beck, J., Benson, D. A., Bollin, C., Bolton, E., Bourexis, D., Brister, J. R., Bryant, S. H., Canese, K., Cavanaugh, M., Charowhas, C., Clark, K., Dondoshansky, I., Feolo, M., Fitzpatrick, L., Funk, K., Geer, L. Y., Gorelenkov, V., … Zbicz, K. (2018). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research*, *46*(D1), D8–D13. https://doi.org/10.1093/nar/gkx1095

Aguillon, S. M., Fitzpatrick, J. W., Bowman, R., Schoech, S. J., Clark, A. G., Coop, G., & Chen, N. (2017). Deconstructing isolation-by-distance: The genomic consequences of limited dispersal. *PLoS Genetics*, *13*(8), 1–27. https://doi.org/10.1371/journal.pgen.1006911

Alexander, D. H., Novembre, J., & Lange, K. (2009). Fast model-based estimation of ancestry in unrelated individuals. *Genome Research*, *19*(9), 1655–1664. https://doi.org/10.1101/gr.094052.109

Ali, O. A., O'Rourke, S. M., Amish, S. J., Meek, M. H., Luikart, G., Jeffres, C., & Miller, M. R. (2016). Rad capture (Rapture): Flexible and efficient sequence-based genotyping. *Genetics*, *202*(2), 389–400. https://doi.org/10.1534/genetics.115.183665

Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*. Retrieved from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Asztalos, M., Glaw, F., Franzen, M., Kindler, C., & Fritz, U. (2021). Transalpine dispersal: Italian barred grass snakes in southernmost Bavaria – This far but no further! *Journal of Zoological Systematics and Evolutionary Research*, *59*(5), 1136–1148. https://doi.org/10.1111/jzs.12471

Beniston, M. (2006). Mountain weather and climate: A general overview and a focus on climatic change in the Alps. *Hydrobiologia*, *562*(1), 3–16. https://doi.org/10.1007/s10750-005-1802-0

Bianchini, G. (2021). *TreeViewer*.

Bie, T., Meester, L., Brendonck, L., Martens, K., Goddeeris, B., Ercken, D., Hampel, H., Denys, L., Vanhecke, L., Gucht, K., Wichelen, J., Vyverman, W., & Declerck, S. A. J. (2012). Body size and dispersal mode as key traits determining metacommunity structure of aquatic organisms. *Ecology Letters*, *15*(7), 740–747. https://doi.org/10.1111/j.1461-0248.2012.01794.x

Blair, C., Campbell, C. R., & Yoder, A. D. (2015). Assessing the utility of whole genome amplified DNA for next-generation molecular ecology. *Molecular Ecology Resources*, *15*(5), 1079–1090. https://doi.org/10.1111/1755-0998.12376

Blattner, L., Ebner, J. N., Zopfi, J., & von Fumetti, S. (2021). Targeted non-invasive bioindicator species detection in eDNA water samples to assess and monitor the integrity of vulnerable alpine freshwater environments. *Ecological Indicators*, *129*, 107916. https://doi.org/10.1016/j.ecolind.2021.107916

Blattner, L., Gerecke, R., & Von Fumetti, S. (2019). Hidden biodiversity revealed by integrated morphology and genetic species delimitation of spring dwelling water mite species (Acari, Parasitengona: Hydrachnidia). *Parasites and Vectors*, *12*(1), 1–13. https://doi.org/10.1186/s13071-019-3750-y

Bohonak, A. J. (1999). Effect of insect-mediated dispersal on the genetic structure of postglacial water mite populations. *Heredity*, *82*, 451–461. https://doi.org/10.1038/sj.hdy.6885050

Borcard, D., Gillet, F., & Legendre, P. (2018). *Numerical ecology with R* (2nd ed.). Springer International Publishing.

Broquet, T., Angelone, S., Jaquiery, J., Joly, P., Lena, J.-P., Lengagne, T., Plenet, S., Luquet, E., & Perrin, N. (2010). Genetic bottlenecks driven by population disconnection. *Conservation Biology*, *24*(6), 1596–1605. https://doi.org/10.1111/j.1523-1739.2010.01556.x

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics*, *10*, 1–9. https://doi.org/10.1186/1471-2105-10-421

Cantonati, M., Angeli, N., Bertuzzi, E., Spitale, D., & Lange-Bertalot, H. (2012). Diatoms in springs of the Alps: Spring types, environmental determinants, and substratum. *Freshwater Science*, *31*(2), 499–524. https://doi.org/10.1899/11-065.1

Cantonati, M., Fensham, R. J., Stevens, L. E., Gerecke, R., Glazier, D. S., Goldscheider, N., Knight, R. L., Richardson, J. S., Springer, A. E., & Tockner, K. (2021). Urgent plea for global protection of springs. *Conservation Biology*, *35*(1), 378–382. https://doi.org/10.1111/cobi.13576

Cantonati, M., Füreder, L., Gerecke, R., Jüttner, I., & Cox, E. J. (2012). Crenic habitats, hotspots for freshwater biodiversity conservation: Toward an understanding of their ecology. *Freshwater Science*, *31*(2), 463–480. https://doi.org/10.1899/11-111.1

Cantonati, M., Gerecke, R., & Bertuzzi, E. (2006). Springs of the Alps – Sensitive ecosystems to environmental change: From biodiversity assessments to long-term studies. *Hydrobiologia*, *562*(1), 59–96. https://doi.org/10.1007/s10750-005-1806-9

Cantonati, M., Segadelli, S., Spitale, D., Gabrieli, J., Gerecke, R., Angeli, N., De Nardo, M. T., Ogata, K., & Wehr, J. D. (2020). Geological and hydrochemical prerequisites of unexpectedly high biodiversity in spring ecosystems at the landscape level. *Science of the Total Environment*, *740*, 140157. https://doi.org/10.1016/j.scitotenv.2020.140157

Cartwright, J. (2019). Ecological islands: Conserving biodiversity hotspots in a changing climate. *Frontiers in Ecology and the Environment*, *17*(6), 331–340. https://doi.org/10.1002/fee.2058

Cartwright, J. M., Dwire, K. A., Freed, Z., Hammer, S. J., McLaughlin, B., Misztal, L. W., Schenk, E. R., Spence, J. R., Springer, A. E., & Stevens, L. E. (2020). Oases of the future? Springs as potential hydrologic refugia in drying climates. *Frontiers in Ecology and the Environment*, *18*(5), 245–253. https://doi.org/10.1002/fee.2191

Chang, C. C., Chow, C. C., Tellier, L. C. A. M., Vattikuti, S., Purcell, S. M., & Lee, J. J. (2015). Second-generation PLINK: Rising to the challenge of larger and richer datasets. *GigaScience*, *4*(1), 1–16. https://doi.org/10.1186/s13742-015-0047-8

Charlesworth, B. (2009). Fundamental concepts in genetics: Effective population size and patterns of molecular evolution and variation. *Nature Reviews Genetics*, *10*(3), 195–205. https://doi.org/10.1038/nrg2526

Cruaud, A., Groussier, G., Genson, G., Sauné, L., Polaszek, A., & Rasplus, J. Y. (2018). Pushing the limits of whole genome amplification: successful sequencing of RADseq library from a single microhymenopteran (Chalcidoidea, *Trichogramma*). *PeerJ*, *6*, e5640. http://doi.org/10.7717/peerj.5640

Dabert, M., Proctor, H., & Dabert, J. (2016). Higher-level molecular phylogeny of the water mites (Acariformes: Prostigmata: Parasitengonina: Hydrachnidia). *Molecular Phylogenetics and Evolution*, *101*, 75–90. https://doi.org/10.1016/j.ympev.2016.05.004

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., Handsaker, R. E., Lunter, G., Marth, G. T., Sherry, S. T., McVean, G., & Durbin, R. (2011). The variant call format and VCFtools. *Bioinformatics*, *27*(15), 2156–2158. https://doi.org/10.1093/bioinformatics/btr330

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., Whitwham, A., Keane, T., McCarthy, S. A., Davies, R. M., & Li, H. (2021). Twelve years of SAMtools and BCFtools. *GigaScience*, *10*(2), 1–4. https://doi.org/10.1093/gigascience/giab008

de Medeiros, B. A. S., & Farrell, B. D. (2018). Whole-genome amplification in doubledigest RADseq results in adequate libraries but fewer sequenced loci. *PeerJ*, *6*, e5089. https://doi.org/10.7717/peerj.5089

Di Sabatino, A., Coscieme, L., Miccoli, F. P., & Cristiano, G. (2021). Benthic invertebrate assemblages and leaf-litter breakdown along the eucrenal–hypocrenal ecotone of a rheocrene spring in Central Italy: Are there spatial and seasonal differences? *Ecohydrology*, *14*, 1–11. https://doi.org/10.1002/eco.2289

Di Sabatino, A., Gerecke, R., Gledhill, T., & Smit, H. (2010). Chelicerata: Acari II. In R. Gerecke (Ed.), *Süsswasserfauna von Mitteleuropa* (p. 234). Spektrum Akademischer Verlag.

Do, C., Waples, R. S., Peel, D., Macbeth, G. M., Tillett, B. J., & Ovenden, J. R. (2014). NeEstimator v2: Re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. *Molecular Ecology Resources*, *14*(1), 209–214. https://doi.org/10.1111/1755-0998.12157

Dray, S., Blanchet, D., Borcard, D., Guenard, G., Jombart, T., Larocque, G., Legendre, P., Madi, N., & Wagner, H. H. (2021). adespatial: Multivariate multiscale spatial analysis. R package.

Eisenhauer, N., Bonn, A., & A. Guerra, C. (2019). Recognizing the quiet extinction of invertebrates. *Nature Communications*, *10*(1), 1–3. https://doi.org/10.1038/s41467-018-07916-1

Elsen, P. R., & Tingley, M. W. (2015). Global mountain topography and the fate of montane species under climate change. *Nature Climate Change*, *5*, 5–10. https://doi.org/10.1038/nclimate2656

Engelhardt, C. H. M., Haase, P., & Pauls, S. U. (2011). From the Western Alps across Central Europe: Postglacial recolonisation of the tufa stream specialist *Rhyacophila pubescens* (Insecta, Trichoptera). *Frontiers in Zoology*, *8*, 1–14. https://doi.org/10.1186/1742-9994-8-10

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics*, *131*(2), 479–491. https://doi.org/10.1093/genetics/131.2.479

Fattorini, S., Borges, P. A. V., Fiasca, B., & Galassi, D. M. P. (2016). Trapped in the web of water: Groundwater-fed springs are island-like ecosystems for the meiofauna. *Ecology and Evolution*, *6*(23), 8389–8401. https://doi.org/10.1002/ece3.2535

Galbreath, K. E., & Cook, J. A. (2004). Genetic consequences of Pleistocene glaciations for the tundra vole (*Microtus oeconomus*) in Beringia. *Molecular Ecology*, *13*(1), 135–148. https://doi.org/10.1046/j.1365-294X.2004.02026.x

Garant, D., Forde, S. E., & Hendry, A. P. (2007). The multifarious effects of dispersal and gene flow on contemporary adaptation. *Functional Ecology*, *21*(3), 434–443. https://doi.org/10.1111/j.1365-2435.2006.01228.x

Gerecke, R. (1993). The water mites of the genus *Partnunia* Piersing, 1896 (Acari, Actinedida, Hydryphantidae). *Entomologica Basiliensia*, *15*, 13–44.

Gerecke, R. (1996). Untersuchungen über die Wassermilben der Familie Hydryphantidae (Acari, Actinedida) in der Westpalaearktis II. Die Wassermilben der Familie Hydryphanidae in den Mittelmeerländern - Systematik, Faunistik, Zoogeographie. *Archiv Für Hydrobiologie Suppl*, *77*, 337–513.

Gerecke, R., Martin, P., & Gledhill, T. (2018). Water mites (Acari Parasitengona: Hydrachnidia) as inhabitants of groundwater-influenced habitats – considerations following an update of Limnofauna Europaea. *Limnologica*, *69*, 81–93. https://doi.org/10.1016/j.limno.2017.11.008

Gerecke, R., Schatz, H., & Wohltmann, A. (2009). The mites (Chelicerata: Acari) of the CRENODAT project: Faunistic records and ecological data from springs in the autonomous province of Trento (Italian Alps). *International Journal of Acarology*, *35*, 303–333. https://doi.org/10.1080/01647950903059452

Gerecke, R., Stoch, F., Meisch, C., & Schrankel, I. (2005). Die Fauna der Quellen und des hyporheischen Interstitials in Luxemburg. *Ferrantia*, *41*, 140.

Gibson, M. J. S., & Moyle, L. C. (2020). Regional differences in the abiotic environment contribute to genomic divergence within a wild tomato species. *Molecular Ecology*, *29*(12), 2204–2217. https://doi.org/10.1111/mec.15477

Glazier, D. S. (2014). *Springs. Reference module in earth systems and environmental sciences*. Elsevier.

Gobiet, A., & Kotlarski, S. (2020). Future Climate Change in the European Alps. In *Oxford Research Encyclopedias*, *Climate Science*. https://doi.org/10.1093/acrefore/9780190228620.013.767

Hare, M. P., Nunney, L., Schwartz, M. K., Ruzzante, D. E., Burford, M., Waples, R. S., Ruegg, K., & Palstra, F. (2011). Understanding and estimating effective population size for practical application in marine species management. *Conservation Biology*, *25*(3), 438–449. https://doi.org/10.1111/j.1523-1739.2010.01637.x

Hewitt, G. (2000). The genetic legacy of the quaternary ice ages. *Nature*, *405*(6789), 907–913. https://doi.org/10.1038/35016000

Hohenlohe, P. A., Funk, W. C., & Rajora, O. P. (2021). Population genomics for wildlife conservation and management. *Molecular Ecology*, *30*(1), 62–82. https://doi.org/10.1111/mec.15720

Huber, K., Jacquet, S., Rivallan, R., Adakal, H., Vachiery, N., Risterucci, A. M., & Chevillon, C. (2019). Low effective population sizes in *Amblyomma variegatum*, the tropical bont tick. *Ticks and Tick-Borne Diseases*, *10*(1), 93–99. https://doi.org/10.1016/j.ttbdis.2018.08.019

Kozarewa, I., & Turner, D. J. (2011). 96-plex molecular barcoding for the Illumina genome analyzer. In Y. M. Kwon, & S. C. Ricke (Eds.), *High-throughput next generation sequencing: Methods and applications* (pp. 279–298). Humana Press.

Kreiner, D., Maringer, A., Remschak, C., Haseke, H., & Gerecke, R. (2018). QUELLEN – Forschung 2012–2017. In *Schriften des Nationalparks Gesäuse* (p. 193). Nationalpark Gesäuse GmbH.

Küry, D., Lubini, V., & Stucki, P. (2016). Temperature patterns and factors governing thermal response in high elevation springs of the Swiss Central Alps. *Hydrobiologia*, *793*(1), 185–197. https://doi.org/10.1007/s10750-016-2918-0

Lai, G. G., Padedda, B. M., Ector, L., Wetzel, C. E., Lugliè, A., & Cantonati, M. (2020). Mediterranean karst springs: Diatom biodiversity hotspots under the pressure of hydrological fluctuation and nutrient enrichment. *Plant Biosystems*, *154*(5), 673–684. https://doi.org/10.1080/11263504.2019.1674402

Lencioni, V., Marziali, L., & Rossaro, B. (2011). Diversity and distribution of chironomids (Diptera, Chironomidae) in pristine Alpine and pre-Alpine springs (Northern Italy). *Journal of Limnology*, *70*, 106–121. https://doi.org/10.3274/JL11-70-S1-08

Lencioni, V., Marziali, L., & Rossaro, B. (2012). Chironomids as bioindicators of environmental quality in mountain springs. *Freshwater Science*, *31*(2), 525–541. https://doi.org/10.1899/11-038.1

Levison, J., Larocque, M., & Ouellet, M. (2014). Modeling low-flow bedrock springs providing ecological habitats with climate change scenarios. *Journal of Hydrology*, *515*, 16–28. https://doi.org/10.1016/j.jhydrol.2014.04.042

Li, H. (2018). Minimap2: Pairwise alignment for nucleotide sequences. *Bioinformatics*, *34*(18), 3094–3100. https://doi.org/10.1093/bioinformatics/bty191

Lucek, K., Butlin, R. K., & Patsiou, T. (2020). Secondary contact zones of closely-related *Erebia* butterflies overlap with narrow phenotypic and parasitic clines. *Journal of Evolutionary Biology*, *33*(9), 1152–1163. https://doi.org/10.1111/jeb.13669

MacArthur, R. H., & Wilson, E. O. (1967). *The theory of island biogeography*. Princeton University Press.

Mahé, F., Czech, L., Stamatakis, A., Quince, C., de Vargas, C., Dunthorn, M., & Rognes, T. (2021). Swarm v3: towards tera-scale amplicon clustering. *Bioinformatics*. http://doi.org/10.1093/bioinformatics/btab493

Manenti, R., & Piazza, B. (2021). Between darkness and light: spring habitats provide new perspectives for modern researchers on groundwater biology. *PeerJ*, *9*, e11711. http://doi.org/10.7717/peerj.11711

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Research*, *27*(2 Part 1), 209–220.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10–12. https://doi.org/10.14806/ej.17.1.200

Martin, P., Koester, M., Schynawa, L., & Gergs, R. (2015). First detection of prey DNA in *Hygrobates fluviatilis* (Hydrachnidia, Acari): A new approach for determining predator–prey relationships in water mites. *Experimental and Applied Acarology*, *67*(3), 373–380. https://doi.org/10.1007/s10493-015-9956-6

Martin, P., & Stur, E. (2006). Parasite-host associations and life cycles of spring-living water mites (Hydrachnidia, Acari) from Luxembourg. *Hydrobiologia*, *573*, 17–37. https://doi.org/10.1007/s10750-006-0246-5

Martin, P., Stur, E., & Wiedenbrug, S. (2009). Larval parasitism of spring-dwelling alpine water mites (Hydrachnidia, Acari): A study with particular reference to chironomid hosts. *Aquatic Ecology*, *44*, 431–448. https://doi.org/10.1007/s10452-009-9301-4

Meirmans, P. G. (2020). genodive version 3.0: Easy-to-use software for the analysis of genetic data of diploids and polyploids. *Molecular Ecology Resources*, *20*(4), 1126–1131. https://doi.org/10.1111/1755-0998.13145

Montero-Pau, J., Gómez, A., & Serra, M. (2018). Founder effects drive the genetic structure of passively dispersed aquatic invertebrates. *PeerJ*, *6*, 1–25. https://doi.org/10.7717/peerj.6094

Mussmann, S. M., Douglas, M. R., Chafin, T. K., & Douglas, M. E. (2019). BA3-SNPs: Contemporary migration reconfigured in BayesAss for next-generation sequence data. *Methods in Ecology and Evolution*, *10*(10), 1808–1813. https://doi.org/10.1111/2041-210X.13252

Myers, M. J., Sperling, F. A. H., & Resh, V. H. (2001). Dispersal of two species of Trichoptera from desert springs: Conservation implications for isolated vs. connected populations. *Journal of Insect Conservation*, *5*(3), 207–215. https://doi.org/10.1023/A:1017998513721

Neel, M. C., McKelvey, K., Ryman, N., Lloyd, M. W., Short Bull, R., Allendorf, F. W., Schwartz, M. K., & Waples, R. S. (2013). Estimation of effective population size in continuously distributed populations: There goes the neighborhood. *Heredity*, *111*(3), 189–199. https://doi.org/10.1038/hdy.2013.37

Nielson, K. G., Gill, K. M., Springer, A. E., Ledbetter, J. D., Stevens, L. E., & Rood, S. B. (2019). Springs ecosystems: Vulnerable ecological islands where environmental conditions, life history traits, and human disturbance facilitate non-native plant invasions. *Biological Invasions*, *21*(9), 2963–2981. https://doi.org/10.1007/s10530-019-02025-6

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B.., Simpson, G. L., Solymos, P., Stevens, M. M. H., Szoecs, E. & Wagner, H. (2020). *Vegan: Community Ecology Package. R package*.

Padgham, M., & Sumner, M. D. (2021). *geodist: Fast, dependency-free geodesic distance calculations*.

Pascual, R., Nebra, A., Gomà, J., Pedrocchi, C., & Cadiach, O. (2020). First data on the biological richness of Mediterranean springs. *Limnetica*, *39*(1), 121–139. https://doi.org/10.23818/limn.39.09

Pauls, S. U., Lumbsch, H. T., & Haase, P. (2006). Phylogeography of the montane caddisfly *Drusus discolor*: Evidence for multiple refugia and

periglacial survival. *Molecular Ecology*, *15*(8), 2153–2169. https://doi.org/10.1111/j.1365-294X.2006.02916.x

Pečnerová, P., Palkopoulou, E., Wheat, C. W., Skoglund, P., Vartanyan, S., Tikhonov, A., Nikolskiy, P., van der Plicht, J., Díez-del-Molino, D., & Dalén, L. (2017). Mitogenome evolution in the last surviving woolly mammoth population reveals neutral and functional consequences of small population size. *Evolution Letters*, *1*(6), 292–303. https://doi.org/10.1002/evl3.33

Peter, B. M., & Slatkin, M. (2015). The effective founder effect in a spatially expanding population. *Evolution*, *69*(3), 721–734. https://doi.org/10.1111/evo.12609

Pinheiro, H. T., Bernardi, G., Simon, T., Joyeux, J.-C., Macieira, R. M., Gasparini, J. L., Rocha, C., & Rocha, L. A. (2017). Island biogeography of marine organisms. *Nature*, *549*(7670), 82–85. https://doi.org/10.1038/nature23680

Pozojević, I., Pešić, V., Goldschmidt, T., & Gottstein, S. (2020). Crenal habitats: Sources of water mite (Acari: Hydrachnidia) diversity. *Diversity*, *12*(9), 19–23. https://doi.org/10.3390/D12090316

R Core Team. (2021). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Rader, R. B., Unmack, P. J., Christensen, W. F., & Jiang, X. (2019). Connectivity of two species with contrasting dispersal abilities: A test of the isolated tributary hypothesis. *Freshwater Science*, *38*(1), 142–155. https://doi.org/10.1086/701671

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, *67*(5), 901–904. https://doi.org/10.1093/sysbio/syy032

Reiss, M., Martin, P., Gerecke, R., & von Fumetti, S. (2016). Limnoecological characteristics and distribution patterns of spring habitats and invertebrates from the Lowlands to the Alps. *Environmental Earth Sciences*, *75*(12), 1–11. https://doi.org/10.1007/s12665-016-5818-8

Rochette, N. C., Rivera-Colón, A. G., & Catchen, J. M. (2019). Stacks 2: Analytical methods for paired-end sequencing improve RADseq-based population genomics. *Molecular Ecology*, *28*(21), 4737–4754. https://doi.org/10.1111/mec.15253

Rogora, M., Frate, L., Carranza, M. L., Freppaz, M., Stanisci, A., Bertani, I., Bottarin, R., Brambilla, A., Canullo, R., Carbognani, M., Cerrato, C., Chelli, S., Cremonese, E., Cutini, M., Di Musciano, M., Erschbamer, B., Godone, D., Iocchi, M., Isabellon, M., … Matteucci, G. (2018). Assessment of climate change effects on mountain ecosystems through a cross-site analysis in the Alps and Apennines. *Science of the Total Environment*, *624*, 1429–1442. https://doi.org/10.1016/j.scitotenv.2017.12.155

Rosati, M., Rossetti, G., Cantonati, M., Pieri, V., Roca, J. R., & Mesquita-Joanes, F. (2017). Are aquatic assemblages from small water bodies more stochastic in dryer climates? An analysis of ostracod spring metacommunities. *Hydrobiologia*, *793*(1), 199–212. https://doi.org/10.1007/s10750-016-2938-9

Sánchez, M. I., Coccia, C., Valdecasas, A. G., Boyero, L., & Green, A. J. (2015). Parasitism by water mites in native and exotic Corixidae: Are mites limiting the invasion of the water boatman *Trichocorixa verticalis* (Fieber, 1851)? *Journal of Insect Conservation*, *19*(3), 433–447. https://doi.org/10.1007/s10841-015-9764-7

Seehausen, O. (2004). Hybridization and adaptive radiation. *Trends in Ecology and Evolution*, *19*(4), 198–207. https://doi.org/10.1016/j.tree.2004.01.003

Sekar, S., & Karanth, P. (2013). Flying between Sky Islands: The effect of naturally fragmented habitat on butterfly population structure. *PLoS One*, *8*(8), e71573. https://doi.org/10.1371/journal.pone.0071573

Sexton, J. P., Hangartner, S. B., & Hoffmann, A. A. (2014). Genetic isolation by environment or distance: Which pattern of gene flow is most common? *Evolution*, *68*(1), 1–15. https://doi.org/10.1111/evo.12258

Sgrò, C. M., Lowe, A. J., & Hoffmann, A. A. (2011). Building evolutionary resilience for conserving biodiversity under climate change. *Evolutionary Applications*, *4*(2), 326–337. https://doi.org/10.1111/j.1752-4571.2010.00157.x

Shama, L. N. S., Kubow, K. B., Jokela, J., & Robinson, C. T. (2011). Bottlenecks drive temporal and spatial genetic changes in alpine caddisfly metapopulations. *BMC Evolutionary Biology*, *11*(1), https://doi.org/10.1186/1471-2148-11-278

Shirk, A. J., Landguth, E. L., & Cushman, S. A. (2021). The effect of gene flow from unsampled demes in landscape genetic analysis. *Molecular Ecology Resources*, *21*(2), 394–403. https://doi.org/10.1111/1755-0998.13267

Smith, B. P. (1988). Host-Parasite Interaction and impact of larval water mites on insects. *Annual Review of Entomology*, *33*, 487–507. https://doi.org/10.1146/annurev.en.33.010188.002415

Spitale, D., Leira, M., Angeli, N., & Cantonati, M. (2012). Environmental classification of springs of the Italian Alps and its consistency across multiple taxonomic groups. *Freshwater Science*, *31*(2), 563–574. https://doi.org/10.1899/10-038.1

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*(9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Stevens, L. E., Schenk, E. R., & Springer, A. E. (2021). Springs ecosystem classification. *Ecological Applications*, *31*(1), https://doi.org/10.1002/eap.2218

Storfer, A., Murphy, M. A., Spear, S. F., Holderegger, R., & Waits, L. P. (2010). Landscape genetics: Where are we now? *Molecular Ecology*, *19*(17), 3496–3514. https://doi.org/10.1111/j.1365-294X.2010.04691.x

Stutz, H. L., Shiozawa, D. K., & Evans, R. P. (2010). Inferring dispersal of aquatic invertebrates from genetic variation: A comparative study of an amphipod and mayfly in Great Basin springs. *Journal of the North American Benthological Society*, *29*(3), 1132–1147. https://doi.org/10.1899/09-157.1

Van Buskirk, J., & Jansen van Rensburg, A. (2020). Relative importance of isolation-by-environment and other determinants of gene flow in an alpine amphibian. *Evolution*, *74*(5), 962–978. https://doi.org/10.1111/evo.13955

Von Fumetti, S., & Blattner, L. (2017). Faunistic assemblages of natural springs in different areas in the Swiss National Park: A small-scale comparison. *Hydrobiologia*, *793*(1), 175–184. https://doi.org/10.1007/s10750-016-2788-5

Waples, R. S., & Do, C. (2008). LDNE: A program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources*, *8*(4), 753–756. https://doi.org/10.1111/j.1755-0998.2007.02061.x

Ward, J. V. (1994). Ecology of alpine streams. *Freshwater Biology*, *32*(2), 277–294. https://doi.org/10.1111/j.1365-2427.1994.tb01126.x

Wilson, G. A., & Rannala, B. (2003). Bayesian inference of recent migration rates using multilocus genotypes. *Genetics*, *163*(3), 1177–1191. https://doi.org/10.1093/genetics/163.3.1177

Winterberg, S., & Willett, S. D. (2019). Greater Alpine river network evolution, interpretations based on novel drainage analysis. *Swiss Journal of Geosciences*, *112*(1), 3–22. https://doi.org/10.1007/s00015-018-0332-5

Woodward, G., Perkins, D. M., & Brown, L. E. (2010). Climate change and freshwater ecosystems: Impacts across multiple levels of organization. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1549), 2093–2106. https://doi.org/10.1098/rstb.2010.0055

Wurzbacher, C., Kreiling, A.-K., Svantesson, S., Van den Wyngaert, S., Larsson, E., Heeger, F., Nilsson, H. R., & Pálsson, S. (2020). Fungal communities in groundwater springs along the volcanic zone of Iceland. *Inland Waters*, *10*(3), 418–427. https://doi.org/10.1080/20442041.2019.1689065

Zawal, A. (2003). The role of insects in the dispersion of water mites. *Acta Biologica Universitatis Daugavpiliensis*, *3*, 9–14.

BIOSKETCH

Lucas Blattner is working in the field of molecular biogeography and is interested in environmental sciences in general. This study is part of his Ph.D. thesis, which focused on studying alpine spring ecosystems by investigating crenobiontic Hydrachnidia species to understand their ecology and mechanisms shaping their distribution.

Author contributions: All authors edited and approved the manuscript. LB conceived and designed the study and wrote the manuscript. He planned and conducted field and laboratory work as well as bioinformatic processing of the data. KL substantially contributed to data analysis, interpretation and manuscript editing. NB was mainly involved in laboratory protocol development and optimization. DB supported the study with conceptual and data analysis input and SF contributed to conceptualization, manuscript editing and study realization.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Blattner, L., Lucek, K., Beck, N., Berner, D., & von Fumetti, S. (2021). Intra-Alpine Islands: Population genomic inference reveals high degree of isolation between freshwater spring habitats. *Diversity and Distributions*, 00, 1–15. https://doi.org/10.1111/ddi.13461

Chapter IV

Targeted non-invasive bioindicator species detection in eDNA water samples to assess and monitor the integrity of vulnerable alpine freshwater environments

Published in *Ecological Indicators* (2021)

Published article on pages: 57 – 66

The article and accompanying supplementary information are available at:

https://doi.org/10.1016/j.ecolind.2021.107916

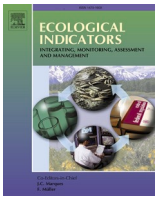
Environmental DNA sampling by filtering water of the Plan da l'Acqua Suot spring in the Swiss National Park.

Ecological Indicators 129 (2021) 107916

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind



Targeted non-invasive bioindicator species detection in eDNA water samples to assess and monitor the integrity of vulnerable alpine freshwater environments

Lucas Blattner [a,*], Joshua Niklas Ebner [a], Jakob Zopfi [b], Stefanie von Fumetti [a]

[a] University of Basel, Department of Environmental Sciences, Geoecology, Klingelbergstrasse 27, 4056 Basel, Switzerland
[b] University of Basel, Department of Environmental Sciences, Aquatic and Isotope Biogeochemistry, Bernoullistrasse 30, 4056 Basel, Switzerland

ARTICLE INFO

Keywords:
eDNA
Bioindicator species
qPCR
Spring Ecosystem
Invertebrates
Non-invasive

ABSTRACT

Due to a high number of specialized species and unique environmental conditions, alpine spring ecosystems are particularly vulnerable to environmental change and human impact. Therefore, the assessment of ecosystem integrity through habitat monitoring over long periods of time is of particular importance, especially in protected areas. Bioindication by conventional ecosystem assessment and monitoring includes sampling whole communities and morphological species identification. This approach, however, brings along major drawbacks such as high invasiveness, low reproducibility, low specificity and is relatively time-consuming. To overcome these issues, we developed a targeted bioindicator species eDNA detection approach for representative freshwater macroinvertebrate species and compared the results with detection through conventional sampling. Macroinvertebrates of 15 springs, located in the Swiss National Park and the UNESCO biosphere reserve Engiadina Val Müstair, were sampled using a hand-net and species were morphologically identified. We selected six spring-bound species: *Hygrobates norvegicus*, *Partnunia steinmanni*, *Dictyogenus fontium*, *Protonemura lateralis*, *Lithax niger,* and *Wormaldia occipitalis* and designed novel, species-specific qPCR primers and hydrolysis probes. Spring eDNA was collected by filtering 1.5 l water through cellulose nitrate filter funnels and DNA extracts were screened by qPCR for the selected bioindicator species. Results showed congruence between conventional and eDNA qPCR-based species detection. The assay targeting *L. niger* was less sensitive and qPCR performance in eDNA samples was decreased compared to the other species, indicating the necessity for careful indicator species choice and evaluation. The newly developed eDNA-based qPCR protocols allow detecting indicator species in alpine springs and represent a non-invasive, sensitive and specific, cost- and time-effective alternative to conventional biomonitoring approaches. Particularly in protected areas such as the Swiss National Park, the implementation of indicator species detection in eDNA filtered water samples can be beneficial and fosters sustainable freshwater ecosystem monitoring and assessment.

1. Introduction

Alpine ecosystems are highly susceptible to environmental change (Beniston, 2006; Fait et al., 2020; Gobiet et al., 2020; Körner, 2003; Mastrotheodoros et al., 2020; Rogora et al., 2018), and the biota of small water bodies are considered to be vulnerable (Oertli et al., 2007; Robinson and Oertli, 2009; Rosati et al., 2017; Rosset et al., 2010). Highly adapted species such as spring-dwelling crenobionts and crenophiles that require oligotrophic and relatively stable environmental conditions are endangered due to global climate change and anthropogenic impacts (Cantonati et al., 2020; Glazier, 2014; Reiss et al., 2016; von Fumetti et al., 2017). The loss of such unique species communities (Cantonati et al., 2020; Pascual et al., 2020; Pozojević et al., 2020) would severely decrease biodiversity and lead to degradation of ecosystem integrity (Eisenhauer et al., 2019; Fanin et al., 2018). Assessment and monitoring of vulnerable ecosystems become increasingly crucial for estimating the impact of environmental change and informing management strategies (Gerecke et al., 2011; Harvey et al., 2020; Küry et al., 2016; Reiss et al., 2016). The development of rapid, cost- and time-effective instruments to assess ecosystem integrity is, therefore, a critical prerequisite advancing environmental monitoring efforts.

Bioindication by recording the presence or absence of species that

* Corresponding author.
E-mail address: lucas.blattner@unibas.ch (L. Blattner).

https://doi.org/10.1016/j.ecolind.2021.107916
Received 4 May 2021; Received in revised form 17 June 2021; Accepted 18 June 2021
Available online 22 June 2021

indicate environmental integrity is widely used as tool to examine the status quo of ecosystems (e.g., Gieswein et al., 2019; Lencioni et al., 2020; Thomsen et al., 2012; Więcek et al., 2013). Particularly freshwater habitats are assessed on a regular basis with standardized bioindication methods that have proven to be reliable and reproducible (Gerecke et al., 2011; Hering et al., 2004; Kuefner et al., 2020; Küry et al., 2016; Morinière et al., 2017; Robinson and Oertli, 2009). Furthermore, national governments (e.g., Switzerland: Lubini et al., 2014; Stucki et al., 2019) and the European Water Framework Directive (European Commission, 2000) have admitted bioindication in freshwater environments as an official tool to assess environmental integrity. However, conventional protocols based on direct sampling, preservation and morphological identification of organisms often require laborious field and laboratory work and are therefore time and cost-intensive (Mächler et al., 2014; Stein et al., 2014; van der Loos and Nijland, 2020; Watts et al., 2019). Furthermore, morphological identification is limited to relatively large specimens in late larval stages (Deiner et al., 2013; Stryjecki et al., 2016; Sweeney et al., 2011), which leads to the inclusion of by-catch species without indicative value that do not contribute to the final ecological assessment (Piper et al., 2019; van der Loos and Nijland, 2020). In conjunction with sampling methods, as e.g., Surber and kick sampling, which disperses large amounts of sediment to retrieve as many species as possible, such techniques can be rather invasive. In small habitats like freshwater springs, invasive sampling methods cause significant disturbances and, when performed regularly or over more extended periods of time, may lead to habitat destruction (Beng and Corlett, 2020; Bossley and Smiley, 2019; Goldberg et al., 2016). Furthermore, undisturbed habitats without direct human impact, and suitable for environmental monitoring, can often be found in protected areas like National Nature Reserves (NNRs) (Robinson and Oertli, 2009; von Fumetti and Blattner, 2016). Paradoxically, invasive and disruptive sampling techniques are common practice and used to assess ecosystems in such areas despite their potential negative influence on natural environments.

The development of alternative methods that overcome these drawbacks have gained increasing attention in studies applying molecular genetic approaches. Collectively, these techniques have multiple advantages compared to conventional bioindicator sampling (Beng and Corlett, 2020; Elbrecht et al., 2017; Liu et al., 2020; Weigand et al., 2019; Zizka et al., 2020). DNA metabarcoding, i.e., high throughput sequencing of PCR amplified DNA extracted from environmental or bulk organismal samples and matching resulting sequences against reference sequence databases of preidentified specimens (Liu et al., 2020), can be used to identify many species in parallel, including life stages that are not distinguishable by morphological species identification (Elbrecht et al., 2017). When applied to environmental DNA (eDNA) samples obtained from filtered water, soil, or sediment instead of bulk macroinvertebrate samples, no living organisms need to be captured, and sampling invasiveness is kept minimal (Beng and Corlett, 2020; Hernandez et al., 2020; Senapati et al., 2019). However, challenges like inconsistent species detection efficiencies due to amplification bias or index jumps (Krehenwinkel et al., 2017; Zinger et al., 2019), incomplete or misleading sequence databases (Elbrecht et al., 2017; Weigand et al., 2019), difficulties to reliably quantify species abundances (Beng and Corlett, 2020; Elbrecht and Leese, 2015), and the need of demanding expert knowledge can diminish the feasibility and implementation in applied environmental monitoring projects.

In recent years, targeted quantitative real-time PCR (qPCR) detection has become a key competence in applied environmental sciences and has found applications in various fields such as, e.g. pathogen detection in wastewater (Langone et al., 2020), monitoring the expansion of invasive species (Adrian-Kalchhauser et al., 2016; Thomas et al., 2019) or quantifying gene expression and adaptation of species to their environment (Evans and Vis, 2020; Zhang et al., 2020). Furthermore, the detection of rare (e.g., Hernandez et al., 2020; Lor et al., 2020) and bioindicator taxa (Senapati et al., 2019) through species and environment-specific qPCR assay establishment gains increasing importance in nature management strategies. Targeted species detection through species-specific qPCR assays can overcome methodological difficulties of metabarcoding, i.e., primer bias, sequencing costs, and laborious data analysis and interpretation. Moreover, amplicon sequencing libraries are usually generated with 30 – 35 PCR cycles (e.g., Gleason et al., 2020; Krehenwinkel et al., 2017; Leese et al., 2021), whereas qPCR assays are commonly conducted with $\pm$ 40 cycles. This increases the amplification performance of low copy number target DNA and consequently the detection sensitivity of low abundand taxa. Therefore, it can be considered an advantageous approach when aiming to detect specific species in freshwater environments.

Given the drawbacks of conventional bioindication methods and the challenges of metabarcoding techniques, we aimed to develop and implement an operational approach to detect macroinvertebrate bioindicator species in protected alpine spring ecosystems. We focused on a set of six representative species and non-invasive eDNA sampling, thus avoiding the necessity to sample living individuals or entire spring communities. The development of a highly specific and sensitive qPCR approach to detect indicator species in filtered water samples circumvents the invasiveness of conventional and laboriousness of metabarcoding techniques without losing the ability to assess bioindicator species presence quantitatively.

2. Materials and methods

2.1. Study sites

The studied springs are located in the southern Swiss Alps as part of the Engiadina Val Müstair UNESCO biosphere reserve (UBEVM) and the Swiss National Park (SNP), which is the biosphere core area and classified as a strict nature reserve (IUCN category Ia). All 15 investigated springs (Fig. 1, Supplementary data 1: Table A1, and Supplementary data 7: KML file) are part of a long-term monitoring program that started in 2019, intending to observe the integrity of spring ecosystems over time and identify possible impacts of climate change. Therefore, spring species communities are sampled and assessed annually in summer, and physicochemical conditions, as well as substrate composition, are documented by using standardized methods according to Hotzy and Römheld, 2008 and Lubini et al., 2014.

2.2. Conventional sampling and indicator species selection

Spring macroinvertebrate communities were sampled in July 2020 by using a hand net (200 μm). Samples were taken at the spring mouth and not >10 m downstream, including all substrate types according to their percental occurrence to account for high habitat diversity. Subsequently, bulk samples containing sediment and organic material were separated into coarse- and fine-grained fractions using a wide-meshed hand net (1 mm). Coarse-grained samples were sorted directly in the field, and large macroinvertebrates preserved in 100% Ethanol. Both fractions were separately transferred to a collecting vessel and preserved with Ethanol (100%) to be transported to the laboratory and sorted under a stereomicroscope. This procedure allows for optimal preservation of intact large specimens suitable for morphological identification and the possibility to screen sediment samples for smaller organisms that can easily be overlooked, such as small Hydrachnidia species.

Morphological identification was done with a focus on the most abundant taxa by using widely applied identification keys (Hydrachnidia: Bartsch et al., 2007; Di Sabatino et al., 2010; Gerecke et al., 2016, Plecoptera: Lubini et al., 2012 and Trichoptera: Waringer and Graf, 2011). Target species with $\leq$ 1 individuals were considered as not detected to compensate for the influence of stochasticity and accidental detection (see Supplementary data 4 Tables D.1 and D.6). The resulting species list reporting presence or absence in all 15 springs was subsequently screened for suitable indicator species by aiming at spring-dwelling species representing the spring fauna of the study area. Due

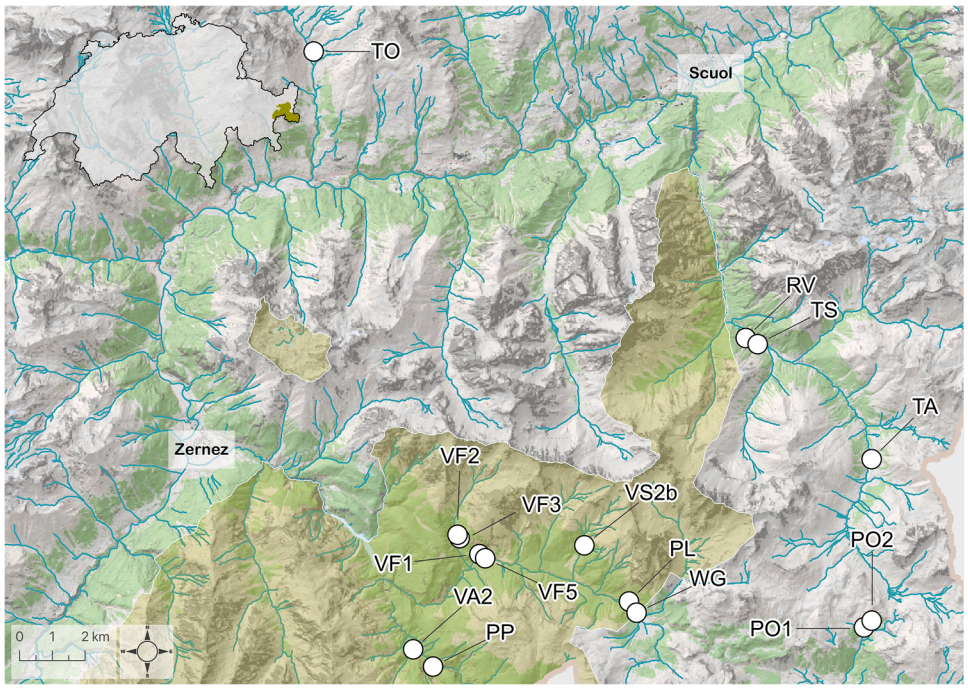


Fig. 1. Map of the study area showing the different springs. The location of the Engiadina Val Müstair UNESCO biosphere reserve (UBEVM) within Switzerland is shown in green colour in the overview that can be found in the top left part of the map. The highlighted part of the map shows the Swiss National Park, which is the core area and most protected part of the biosphere reserve. The map was created in QGIS V3.18 with data of the Swiss Federal Office of Topography, swisstopo, freely available on https://www.swisstopo.admin.ch. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to relatively high species diversity, a high number of spring-bound species, and generally high abundances, we focused mainly on Trichoptera, Hydrachnidia and Plecoptera (Cantonati et al., 2012, 2006; Küry et al., 2016). Furthermore, Trichoptera and Plecoptera species are among other taxa usually targeted in freshwater ecosystem bioindication methodologies (Blackman et al., 2019; Bush et al., 2019; Jourdan et al., 2018; Küry et al., 2016) and Hydrachnidia are known to be exceptionally diverse, with many species that are strictly bound to springs (Blattner et al., 2019; Gerecke et al., 2018). Therefore, these species can potentially be used as powerful bioindicators (Di Sabatino et al., 2003; Gerecke and Lehmann, 2005; Goldschmidt, 2016; Miccoli et al., 2013). Suitable species were chosen according to different criteria, namely: High degree of spring specialization, spring type preference, wide distribution range in the study area, co-occurrence in springs and indicative value according to existing published (e.g. Blattner et al., 2019; Nadig, 1942; von Fumetti and Blattner, 2016; Von Fumetti and Felder, 2014) and unpublished data of the study area. The resulting subset of species: *Lithax niger* (Hagen, 1859), *Wormaldia occipitalis* (Pictet, 1843), *Partnunia steinmanni* Walter, 1906 genotype A and *Hygrobates norvegicus* (Thor, 1897) genotype B sensu Blattner et al., 2019, *Dictyogenus fontium* (Ris, 1896) and *Protonemura lateralis* (Pictet, 1836) were considered as suitable indicator species and candidates for the development of the eDNA-based qPCR assay.

2.3. Sequence library construction

Because of the sparsity of spring species DNA sequences in preexisting databases, we build a custom sequence library to provide the genetic basis for qPCR primer and probe design. Due to the advantages of targeting mitochondrial marker regions for eDNA species detection (Tsuji et al., 2019), mainly because of higher molecule abundance and mutation rate compared to nuclear DNA (Goldberg et al., 2016; Handley, 2015), genetic sequence libraries containing Cytochrome *c* oxidase I (MT-CO1), mitochondrial cytochrome *b* (MT-CYB), mitochondrial 12S (MT-RNR1) and 16S rRNA (MT-RNR2) gene fragments of the target indicator species and as many co-occurring species as possible were generated. Several individuals of different sampling locations per target species were included to address the relatively high variability of mitochondrial DNA and the possibility of geographic genetic separation, which could lead to false negative detection (Arabi et al., 2012; Bergsten et al., 2012; Boumans et al., 2016; Ketchum et al., 2009; Toews and Brelsford, 2012; Weiss and Leese, 2016). Crenobiont species are generally not well studied by molecular methods, and sequence information is scarce or nonexistent. Therefore, a new set of taxon-specific primer pairs (Table 1) targeting the four regions of interest were designed by aligning in-house generated genetic data and sequence information available on NCBI GenBank, including published Plecoptera (Accession no.: KF484757, MG910457, MH085453, MK290826 and MN400756), Trichoptera (Accession no.: KX385010, MG201852, MG669125 and MG980616), and Hydrachnidia (Accession no.: EU856396, LC552026, HQ386015 and MG701313) mitochondrial genomes. The alignments were computed by the MAFFT (Katoh and Standley, 2013) algorithm implemented in Geneious Prime 2021.0.3 (https://www.geneious.com), and all PCR primer pairs were designed by hand, synthesized, and HPLC purified by Microsynth AG (Balgach, Switzerland). To be able to use universal sequencing primers for all reactions, amplification primers were tailed with modified M13 sequences (M13: 5′-TGT AAA ACG ACG GCC AG-3′ and M13r: 5′-CAG GAA ACA GCT ATG AC-3′) (Messing, 1983).

Subsequently, genomic DNA (gDNA) of sampled target and co-occurring species (Supplementary data 2: Tables B.1 and B.2) was extracted by using the magnetic bead-based DNAdvance Kit (Beckman Coulter Live Sciences, Indianapolis USA) according to manufacturer protocol, with 100 µl elution buffer. All PCR amplifications were performed with 0.25 µl of Phusion™ High-Fidelity DNA Polymerase [2 U/µl] (ThermoFisher Scientific, Massachusetts, USA), 5 µl of 5X Phusion™ HF Buffer (ThermoFisher), 0.5 µl of 10 mM dNTPs (SigmaAldrich, Switzerland), 1.25 µl of forward and reverse primers [10 µM each], 5 µl template DNA and molecular grade H_2O to a final reaction volume of 25 µl. PCRs were run with an initial denaturation of 30 s at 98 °C; 35 cycles of 10 s at 98 °C, 30 s at primer pair-specific annealing temperatures (Table 1), and 30 s at 72 °C followed by a final elongation step at 72 °C for 2 min. PCR products were purified enzymatically with ExoSAP-IT™ (Affymetrix Inc., USA) according to manufacturer protocol, and Sanger sequenced bi-directionally with the mentioned M13 primers by Microsynth AG. After sequence retrieval, raw bi-directional reads were

Table 1
Mitochondrial DNA library generation primers designed and used in this study. Oligonucleotide types are abbreviated as F = Forward primer and R = Reverse primer.

Locus	Taxon	Oligonucleotide	Sequence [5′ – 3′]	App. Fragment Length [bp]	Annealing [°C]
MT-CO1	Hydrachnidia	CO1_Hy_nF	CMAAYCAYAAAGAYATTGGAAC	1000	53
		CO1_Hy_R	GCGAAWACWGCTCCTATWG		
	Plecoptera	CO1_Pl_F	ACAAAYCAYAARGAYATTGGAAC	1500	54
		CO1_Pl_R	GRGCTTAAATCCATTGCAC		
	Trichoptera	CO1_Pl/Tr_F	MAAAGAAACTAATGAAYAAACC	1100	52
		CO1_Tr_R	GCRAAWACWGCYCCTATWG		
MT-CYB	Hydrachnidia	CYB _Hy_F	CAAATATCYTTYTGAGGRGC	900	53
		CYB _Hy_R	ATGTACATATCGCCCGTC		
	Plecoptera	CYB _Pl_F	MAAAGAAACTAATGAAYAAACC	1500	52
		CYB _Pl_R	CTTATGYTTTCAAAACATATGC		
	Trichoptera	CYB _Tr_F	AGGWCAAATATCHTTTTGAGG	900	53
		CYB _Tr_R	TTAAGTTTTCAAAACAAAWGCT		
MT-RNR1	Hydrachnidia	12S_Hy_F	GYGACGGGCGATATGTAC	400	60
		12S_Hy_R	AGCAGTTGCGGTTATACG		
	Plecoptera	12S_Pl_F	YCTACTATGTTACGACTTATCTC	400	53
		12S_Pl_R	ATTTGGCGGTGTTTTAGTC		
	Trichoptera	12S_Tr_F	CTACTWTGTTACGACTTRTYTY	500	53
		12S_Tr_R	ARACTRGGATTAGATACCC		
MT-RNR2	Hydrachnidia	16S_Hy_F	TTAYGCTGTTATCCCTTARG	1100	53
		16S_Hy_R	AGGTATGARCCCRTTAGC		
	Plecoptera	16S_Pl_F	TCTATAGGGTCTTCTCGTC	1000	53
		16S_Pl_R	GAGATAAGTCGTAACATAGTAG		
	Trichoptera	16S_Tr_F	AGATAGAAACCAACCTGGC	500	53
		16S_Tr_R	GGTYTGAACTCAGATCATG		

assembled, remaining primer sequences trimmed and final contigs checked for the presence of low quality or ambiguous base calls in Geneious Prime. All sequences are stored and available on NCBI GenBank under accession numbers listed in Supplementary data 2: Tables B.1 and B.2.

2.4. In silico development of qPCR primers and probes for selected bioindicator species

Due to the high specificity and possibility to multiplex several targets in a single reaction (Rodríguez et al., 2015; Tsuji et al., 2019, 2018), a hydrolysis probe-based approach was developed by designing indicator species-specific primer/probe sets. To avoid false positive detections the sequences generated during the sequence library development (Section 2.3) containing target species sequences (Supplementary data 2: Table B.1) as well as sequences of co-occurring macroinvertebrates (Supplementary data 2: Table B.2) were aligned and screened for regions that are highly specific to the target indicator species but as distinct as possible from non-target species. The oligonucleotides were designed according to general recommendations (e.g. Bustin et al., 2020, 2009; Rodríguez et al., 2015) and with at least two of the oligonucleotides having at least two mismatches with non-targets as recommended by Currier et al. (2018). In addition, primers with multiple polymorphisms at the 3′ end between sequences of target and non-target species were preferred as this significantly reduces the possibility of unwanted primer annealing and elongation (Stadhouders et al., 2010; Wilcox et al., 2013). We aimed at theoretical melting temperatures (T_m) of primers as close as possible to 60–65 °C and probes 6–8 °C higher to be able to amplify different templates with standardized qPCR conditions. Furthermore, an amplicon length of < 200 bp was preferred because of the high level of DNA degradation that can be expected from eDNA samples (Mächler et al., 2014; Tsuji et al., 2019; Wood et al., 2020). Several possible primer and probe sets were designed using Primer3 v4.1.0 (Kõressaar et al., 2018; Koressaar and Remm, 2007; Untergasser et al., 2012) and adjusted by hand if necessary. All hydrolysis probes were synthesized with 6-FAM fluorescein at the 5′ end and double quenched with BMN-Q535 between the 8th and 9th base and at the 3′ end, respectively. Final qPCR oligos were ordered with HPLC purification and manufactured by Biomers GmbH (Ulm, Germany).

2.5. In vitro oligonucleotide efficiency and specificity evaluation

Evaluation of possible primer/probe sets was performed in several steps following MIQE guidelines (Bustin et al., 2009), general qPCR recommendations (Taylor et al., 2019), and suggestions from eDNA-specific studies (Blackman et al., 2020; Klymus et al., 2020b; Mauvisseau et al., 2019; Wilcox et al., 2013). First, synthetic standard DNA templates were generated by ordering the target DNA fragments as gBlocks™ (Integrated DNA Technologies Inc., Iowa, USA) (Supplementary data 6 Tables F.1), allowing for precise copy number calculation based on the molecular weight of the DNA fragments supplied by the manufacturer. A 6-fold 1:5 dilution series of these DNA templates was prepared to generate standard curves to test the performance of the designed primer/probe sets. Primer/probe combinations with amplification efficiencies < 90% and showing $r^2 \leq 0.98$ were considered suboptimal and were therefore excluded.

Specificity was evaluated by designing mock communities consisting of gDNA of co-occurring species (Supplementary data 2: Table B.2) in equimolar ratios, resulting in an artificial DNA template containing 0.5 ng DNA of each species. All oligonucleotide sets were tested by amplifying these artificial communities with and without the target species DNA to test for false positive and false negative detection. Only primer/probe sets that did not fail to comply with these specificity tests were considered suitable for the final qPCR eDNA bioindication assay. The sensitivity of each oligonucleotide set was evaluated by calculating the limit of detection (LOD) and the limit of quantification (LOQ). For this, eight replicate amplifications of the 6-fold dilution series were established. LOD was considered as the lowest initial amount of target sequence copies per reaction with 95% detection probability, whereas LOQ was calculated as the lowest amount of sequence copies per reaction with a coefficient of variation (CV) < 35%, which are commonly used thresholds (Conte et al., 2018; Klymus et al., 2020a, 2020b). Target species-specific LOD and LOQ values were calculated using the LOD/LOQ calculator script published by Klymus et al. (2020a) in R (R Core Team, 2017).

All qPCR reactions were run on a MIC magnetic induction cycler (Bio Molecular Systems, Upper Coomera, Australia), using the SensiFAST™ Probe No-ROX Kit (Meridian Live Science, Inc., Tennessee, USA) and identical amplification conditions. The 20 µl final reactions consisted of 10 µl SensiFAST Probe No-ROX Mix [2X], 800 nM (0.8 µl) forward and

reverse primer [20 μM each], 100 nM (0.2 μl) hydrolysis probe [10 μM], 5 μl DNA standard template and 3.2 μl molecular grade H_2O. Reactions were run using the standard SensiFAST two-step amplification protocol, with an initial polymerase activation at 95 °C for 5 min, followed by 40 cycles of 10 s denaturation at 95 °C and 30 s of combined annealing and extension at 60 °C, with fluorescent signal acquisition at the end of each cycle. No template controls (NTC) containing molecular grade water instead of template, negative controls with mock communities lacking DNA of target species, as well as positive controls containing gDNA of the target species were included as duplicates in each run. The qPCR baseline threshold was calculated with the MIC software by implementing dynamic baseline correction with a fluorescence cutoff level of 5% and ignoring the first 5 amplification cycles.

2.6. Environmental DNA (eDNA) sampling

Spring eDNA was collected in July 2020, parallel to the conventional hand-net sampling. At each spring, 1.5 l water per sample was filtered through 250 ml Nalgene™ Single Use Analytical Filter Funnels with 0.45 μm cellulose nitrate membranes (ThermoFisher Scientific) by using a Nalgene™ Polypropylene Vacuum Flask (ThermoFisher Scientific) and a hand-operated vacuum pump. Each sample was composed of six 250 ml sub-samples taken directly at the stream surface between the spring mouth and 10 m downstream. Subsequently, filters were transferred to 2 ml safe-lock tubes (Eppendorf AG, Hamburg, Germany) with tweezers and directly put on dry ice for optimal DNA preservation before storage at − 20 °C in the laboratory until further processing. We considered all material that came into contact with the filtered water as contaminated and discarded it after each sampling. Tweezers were cleaned before and after each filter transfer by flame sterilization with 100% Ethanol and NaOCl (13%) treatment. We sampled three biological replicates per spring and included a negative field sample by filtering tap water brought to the field to test for possible false positive detection caused by cross-contamination during fieldwork.

2.7. eDNA extraction and purification

Due to the possibility of PCR inhibitor presence in eDNA samples (Deiner et al., 2015; Jane et al., 2015; Schabacker et al., 2020), we extracted and purified the DNA captured on filter membranes with the FastDNA Spin Kit for Soil (MP Biomedicals LLC, Santa Ana, USA), which involves a powerful inhibitor removal step. To be able to perform enzymatic cell lysis, which has proven to be beneficial to extract eDNA (Deiner et al., 2015; Deiner and Altermatt., 2014; Wong et al., 2020), instead of the initial mechanical bead beating used by default during the FastDNA Spin Kit procedure, the first steps of the manufacturer protocol were modified. The filter samples were cut into small pieces and transferred to 2 ml tubes containing 900 μl ATL (Qiagen, Hilden, Germany) lysis buffer and 100 μl Proteinase K solution [> 600 mAU/ml] (Qiagen). All samples were then incubated at 56 °C for 48 h to ensure sufficient lysis before continuing with the FastDNA Spin Kit manufacturer protocol. Purified eDNA was subsequently stored at 4 °C until running the qPCR assays, and subsequently at −20 °C for long term storage.

2.8. Indicator species detection and inhibitor control

To monitor possible false-negative indicator species detection caused by qPCR inhibition present in the eDNA templates, we screened all samples by spiking-in an internal positive amplification control (IPC) directly into the qPCR reactions. A pre-established, artificial IPC template and oligonucleotide set, qPCR DNA Extraction and Inhibitor Control Cy®5-QXL®670 (Kaneka Eurogenetec S.A., Seraing, Belgium), was used, and interference of the IPC and the indicator species assays was evaluated in vitro by amplifying the previously mentioned mock community assay (Section 2.5) as negative control and target species gDNA as positive control before continuing with eDNA samples. The IPC assay was used according to the manufacturer's protocol, and a 1:10′000 diluted IPC DNA template was added directly into the reactions to aim for a quantification cycle (Cq) = 30 – 33.

The IPC evaluation reactions as well as all final eDNA indicator species detection reactions were run in duplicates in 20 μl total reaction volume containing 10 μl SensiFAST Probe No-ROX Mix [2X], 800 nM (0.8 μl) forward and reverse primer [20 μM each], 100 nM (0.2 μl) hydrolysis probe [10 μM], 0.2 μl IPC template [1:10′000], 2 μl IPC mix [10X], 1 μM (1 μl) BSA [20 mg/ml] and 5 μl DNA template. Amplification cycle conditions were identical to those in section 2.5, and to monitor potential technical issues, we additionally included NTC negative and positive control reactions in duplicates by replacing the DNA template. eDNA samples that showed qPCR inhibition in the first screening were additionally treated with OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, USA) that has been shown to work well for post-extraction secondary cleanup of eDNA (Mächler et al., 2016; Sanches and Schreier, 2020; Williams et al., 2017). To ensure highly supported positive species detections, only samples with at least 2 out of 3 biological and all technical replicates showing target species amplification were considered positively detected to minimize the possibility of false positives (Supplementary data 4: Tables D.1 and D.6).

3. Results

3.1. Conventional indicator species detection

Conventionally sampled and identified macroinvertebrates (Supplementary data 3: Table C.1) showed species assemblages typical of alpine springs in the area. We recorded 52 Hydrachnidia, Plecoptera, and Trichoptera taxa and we were able to identify 36 of them morphologically to species level. Individuals that were either underdeveloped, highly damaged, or too laborious to identify were assigned to the highest taxonomic resolution possible. With 15 identified species, Trichoptera showed the highest species diversity, followed by Hydrachnidia (11) and Plecoptera (9). The indicator species chosen for the eDNA assay development showed relatively high abundances (reported in Supplementary data 4: Tables D.1 and D.2), especially *P. lateralis* (15 of 15 springs), *D. fontium* (10 springs), *L. niger* (10 springs) and *P. steinmanni* (8 springs) were widely distributed in the study area. *H. norvegicus* B and *W. occipitalis* showed the narrowest distributions and occurred in only 2 and 3 springs, respectively (Supplementary data 4: Tables D.1 and D.6).

3.2. Indicator species targeted qPCR assay development

All final indicator species-specific qPCR primer/probe sets (Table 2) showed no cross-amplification with possible co-occurring non-target species, which was tested with the artificially designed mock communities (Supplementary data 2: Table B.2 and Supplementary data 3: Table C.1). The newly developed primer/probes and amplification conditions can therefore be considered as highly specific. Mean amplification efficiencies of all primer/probe sets were $\geq$ 90% and R^2 values $>$ 0.99, indicating overall good amplification performance (Table 2). The highest LOD was measured for the *Lithax niger* oligonucleotide set, approximately seven times higher compared to *D. fontium* and *P. lateralis* (Table 2). Limits of quantification of the two Hydrachnidia species were considerably higher compared to other taxa (Table 2). All measured standard concentrations, efficiencies, and R^2 values are reported in Supplementary data 5: Tables E.1.

3.3. eDNA indicator species detection

qPCR targeted assays of the spring eDNA samples showed mean reaction efficiencies of 80% (*L.niger & W.occipitalis*), 90% (*H.norvegicus* B & *P.steinmanni* A) and 100% (*D.fontium*) (Supplementary data 4: Tables D.1 and D.6). We measured eDNA target fragment presence

Table 2
eDNA assay qPCR oligonucleotides designed and used in this study. Oligonucleotide types are abbreviated as F = Forward primer, R = Reverse primer and P = Hydrolysis Probe. All probes were labeled with 6-fam fluorophore at the 5′, BMN-Q535 internal quencher between the 8th and 9th base and BMN-Q535 quencher at the 3′ end.

Species	Oligo-nucleotide	Sequence [5′ – 3′]	Fragment Length [bp]	Efficiency [mean ± SD]	R^2 [mean ± SD]	LOD [cp/reaction]	LOQ [cp/reaction]
Hygrobates norvegicus B	Hn_CYB_F	GTTGTGGCCTTGGTCATAAG	124	1.00 ± 0.05	0.998 ± 0.001	15.01	107
	Hn_CYB_R	GGGTTCAATAGGCTTGGC					
	Hn_CYB_P	AATAACTAGTAGCTGTAAATTTAGCCCTCTT					
Partnunia steinmanni A	Ps_CYB_F	TTGGGATTACAGCTGTAG	83	0.94 ± 0.06	0.999 ± 0.006	18.75	288
	Ps_CYB_R	TGTGGATAATGACTATTATTGA					
	Ps_CYB_P	TCAGTAAGAAATGCTACCTTAAATCGT					
Dictyogenus fontium	Df_ CYB _F	GATCTCGCTTTCACCAGC	80	0.97 ± 0.03	0.998 ± 0.001	< 4.83	16
	Df_ CYB _R	CAGTGTGRAGGTAAAGGCAG					
	Df_ CYB _P	CCTCCATGCCAACGGAGCATC					
Protonemura lateralis	Pl_CO1_F	TTCGGTAACTGACTAGTTCCAT	94	0.90 ± 0.02	0.999 ± 0.001	< 6.15	9
	Pl_CO1_R	CAGCTCCATTTTCGACAAGA					
	PL_CO1_P	TGGAGCTCCAGATATGGCATTC					
Lithax niger	Ln_CYB_F	TTAGGGTTAGATTTAGTTCAGTG	154	0.96 ± 0.04	0.999 ± 0.001	38.32	51
	Ln_CYB_R	TGAAATGGGATTTTATCGGA					
	Ln_CYB_P	CTCCATCAAACCGGATCTTCTAACC					
Wormaldia occipitalis	Wo_CYB_F	TTCAGCTATTCCTTATGCA	84	0.98 ± 0.04	0.999 ± 0.001	< 5.17	20
	Wo_CYB_R	TAGCTATGATAATAAATGGAAG					
	Wo_CYB_P	TTTGCTATTGAAAATGCCACTT					

between 80 mean copies/reaction for *Lithax niger* and up to 4089 mean copies/reaction when amplifying *Protonemura lateralis* eDNA (Supplementary data 4: Tables D.3 and D.5). The inhibitor control assay showed overall good sample purity. All replicates of samples VS and VA2 showed no IPC amplification during the first screening, indicating the presence of PCR inhibition, and therefore the respective samples were purified with an additional inhibitor removal step. Subsequent IPC amplification showed successful inhibitor removal.

3.4. Conventional vs. eDNA indicator species detection

Comparison between the two indicator species detection approaches showed good overall accordance (Fig. 2). The Hydrachnidia species detection of *H. norvegicus* B and *P. steinmanni* A is congruent with no differences between conventional and eDNA detection when excluding the cases were detection showed low support. For *D. fontium* and *P. lateralis,* a single spring exhibited positive eDNA detection but absence in the conventional samples. In both cases, conventional sampling recorded only single individuals (Supplementary data 4: Tables D.5 and D.6), which were detected with low support due to disagreement with our dection criterion for bioindication purposes. In two springs (PP and VF5), *W. occipitalis* was recorded in the conventional sampling but failed the criterion of >1 individual whereas the qPCR assay detection passed the validation criteria and reliably detected the species in the eDNA samples (Fig. 2). Furthermore, *W. occipitalis* was recorded by qPCR in sample TO but only one of two biological replicates showd positive

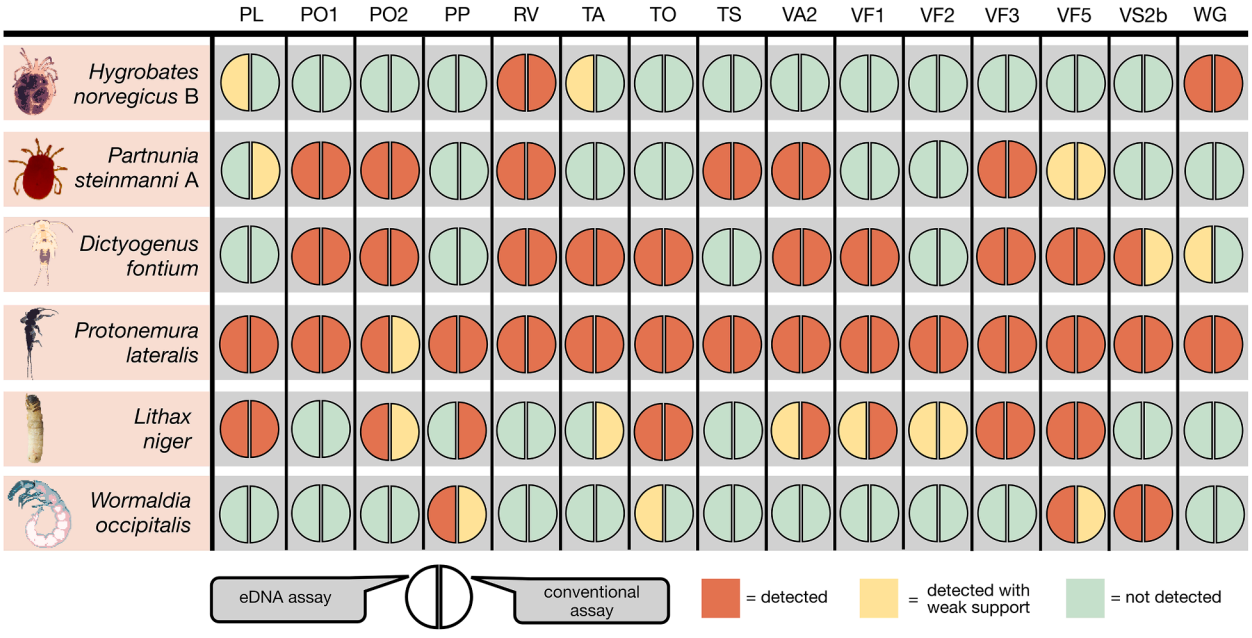


Fig 2. Comparison between conventional and eDNA qPCR bioindicator species detection for 15 freshwater springs in the Swiss National Park. Springs, where the species detection passed our limit of detection criteria for the conventional or the qPCR assay-based approaches, are indicated in red as *detected.* Cases where the species were recovered but the supported detection criteria were not met are shown in yellow as *detected with weak support.* The third category, in light green, shows cases that lack a positive detection of the respective species entirely. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amplification (Supplementary data 4: Table D.4). *Lithax niger* detection showed the highest disagreement between conventional and eDNA qPCR assays. In one out of 15 springs, eDNA detection was positive, contradicting the conventional sampling that indicated the absence of *L. niger*. The qPCR indicator species detection in eDNA samples showed a higher rate of supported detections (41 out of 90 samples) than the conventional approach (39 out of 90 samples), indicating higher overall sensitivity.

4. Discussion

We developed eDNA detection assays for targeting six spring-bound macroinvertebrate species and compared detection rates with the conventional biomonitoring approach. The target spring species that were chosen are widely distributed in the Swiss National Park and have been recorded in previous studies (Robinson et al., 2008; von Fumetti and Blattner, 2016; von Fumetti and Felder, 2014). All investigated springs are inhabited by at least 2 of the indicator species belonging to different taxa. Given the high degree of spring specialization and the presence of multiple species per spring, we consider this species choice as suitable for bioindication and monitoring of undisturbed alpine springs, especially in and around the studied area. Recording the disappearance of these species during the ongoing spring monitoring projects would show deviation from the current natural state of the protected spring habitats and most probably indicate the influence of changed environmental conditions.

The conventional approach is based on sampling whole macroinvertebrate communities and morphologically identifying specimens to species level, which is currently seen as standard spring ecosystem assessment methodology in Europe (e.g., Germany: Hotzy and Römheld, 2008, Switzerland: Lubini et al., 2014). Furthermore, this method is traditionally applied in freshwater assessments (Elbrecht et al., 2017). Morphological species identification of many taxa is restricted to late larval stages with sufficiently developed characters and intact specimens (Deiner et al., 2013; Meredith et al., 2019), resulting in relatively high proportions of unidentifiable individuals. Therefore, our conventional sampling dataset contained specimens that were assigned to genus or family level and, especially the identification of Plecoptera species is often restricted to well-developed larvae (Lubini et al., 2012). Accordingly, specimens potentially belonging to *D. fontium* presumably have been assigned to Perloidea Superfamily, and *P. lateralis* is very likely to be included in *Protonemura* sp., resulting in false-negative indicator species detection. Furthermore, detecting singleton individuals could potentially lead to false positives due to accidental incorporation of specimens during field or laboratory work. This, e.g., can arise because of the occasional occurrence of few individuals in springs that naturally lack established populations or incorrect morphological identification. Particularly when analyzing highly abundant species with populations consisting of numerous individuals, singleton detections should be addressed and discussed carefully (Meredith et al., 2019). In our case, we decided to classify them as detected with weak support and therefore putative false positives, which we consider as appropriate when using the method for bioindication purposes that directly influence management strategies. However, the appropriateness of implementing such a criterion to validate conventional indicator species detection needs further research and should be evaluated specific to the method used and the environment under consideration.

We initially in silico evaluated four different mitochondrial gene fragments (CO1, CYB, 12S and 16S rRNA) for sequence library generation and subsequent qPCR primer/probe design. 12S showed high variability and degree of sequence and length polymorphisms between and within species leading to a decreased suitability for species-specific qPCR primer/probe design. In contrast, the 16S fragment dataset was relatively uniform and, therefore, likewise not ideal. CO1 and CYB, which we recommend for designing indicator species-specific qPCR assays, exhibited the best applicability. The newly designed qPCR primer/probe sets reliably amplified target species DNA with high specificity and sensitivity. Results show that the theoretical limit of detection of *Lithax niger* (38.32 copies/reaction) is higher compared to the other species (e.g., $\leq$ 4.83 copies/reaction for *D. fontium*), which could lead to false negative detection in springs with low abundances but decreases false positive detection probability. However, if qPCR is used to detect the presence of indicative species in eDNA, it is not recommended to entirely rely on theoretical LOD calculations and discard detections in samples below LOD. This is due to the high specificity of hydrolysis probe-based amplification detection, and even quantifications below LOD may be recorded as present (Klymus et al., 2020a).

We generally documented slightly decreased qPCR reaction performance applying the same amplification conditions as used during the in vitro marker development and evaluation compared to filtered water samples. eDNA is of high complexity and contains various types of reaction inhibitors, sources of DNA, and DNA degrading compounds that can influence reaction efficiency (Hunter et al., 2019; Sanches and Schreier, 2020; Wood et al., 2020). Even though we thoroughly evaluated inhibitor presence in our samples using an IPC and applied secondary inhibitor removal as recommended when processing eDNA (Sanches and Schreier, 2020), the decreased reaction performance is likely to result from remaining inhibitors, degraded, or low amounts of intact DNA. However, minor performance losses are expected in such sample types and do not affect indicator species detection (Klymus et al., 2020b). In contrast, accurate quantification is directly affected and reactions with efficiencies that are not close to 100% should be analyzed with care if quantification is the main goal (Klymus et al., 2020b; Taylor et al., 2019). This occured when amplifying *L. niger* and *W. occipitalis* eDNA. Therefore, we do not recommend using these assays if accurate quantification is of importance. Furthermore, our results show high quantification variability between sample replicates, e.g. ranging between 10^1 and 10^3 copies/reaction for *D. fontium* in the VF3 spring. Therefore, a thorough establishment of standardized spring eDNA sampling protocols would be crucial to increase the reproducibility of quantification between sample replicates (Deiner and Altermatt., 2014).

Comparison of both indicator species detection methodologies applied to the 15 investigated springs showed good overall congruence. However, as in the example of the PO2 spring, *P. lateralis* was detected solely by qPCR and the same was observed for *D. fontium* in spring VS2b. In both cases, the conventional species detection was considered negative due to singleton individuals recorded in the samples, which we considered as putative false-positives. This contradiction indicates that either the conventional sampling at this site was biased or that our criterion of classifying single individuals as false positive detections in the conventional approach needs to be reconsidered, which would be adequate for rare species (Meredith et al., 2019). Our experience shows that generally high numbers of *P. lateralis* individuals can be found in springs. Therefore, we assume a biased conventional sampling in this case. Furthermore, we also recorded the opposite situation, where the qPCR assay failed at reliably detecting an indicator species compared to the conventional approach as e.g. *l. niger* results report in VA2 and VF1. Apart from that, *L. niger* showed the most discrepancies between conventional and eDNA-based qPCR detection and the most putative false positives, indicating that the suitability of this species for bioindication purposes may need further research compared to the other spring invertebrates. The slightly decreased qPCR efficiency could be addressed by modifying the primer/probe set or testing an alternative target locus if accurate quantification is requested but with the aim of bioindication not required. Overall, qPCR detection in eDNA samples showed a generally higher detection sensitivity when excluding detections with low support compared to the conventional approach supporting the robustness of our newly developed assay.

We conclude that detecting bioindicator species through targeted qPCR in environmental DNA water samples is a an effective tool that can be used in alpine spring ecosystem assessments. Furthermore, it is a powerful supplement to conventional methodologies and can overcome

their relative drawbacks. The main advantage is the possibility to test the quality of detection rates by biological and technical replication, performance evaluation, and high specificity, enabling the prevention of false negative and false positive indicator species detection. Such bio-indication assays can similarly be developed for various ecosystems (Beng and Corlett, 2020; Liu et al., 2020; Thomsen and Willerslev, 2015). Especially in freshwater habitats located in protected areas, eDNA approaches can serve as a completely non-invasive monitoring tool to investigate ecosystem integrity over long periods of time. As shown by our results, one of the major challenges when developing targeted eDNA detection assays is the choice of suitable indicator species that should be based not only on the organism's ecological prerequisites but also on qPCR specific aspects and a thorough method evaluation.

CRediT authorship contribution statement

Lucas Blattner: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Visualisation, Writing - Original Draft, Project Administration. **Joshua Niklas Ebner:** Conceptualization, Visualization, Writing - Review & Editing. **Jakob Zopfi:** Methodology, Resources, Writing - Review & Editing. **Stefanie von Fumetti:** Investigation, Resources, Data Curation, Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Field sampling coordination and project approval were granted through the Swiss National Park (Dr. S. Wipf), the UNESCO biosphere reserve Engiadina Val Müstair (Dr. A.Abderhalden), and the Swiss Academy of Science (SCNAT). This work was supported by the Swiss National Science Foundation (SNSF) [grant number 31003A_176234]. Furthermore, we want to thank Nathanael Beck, who contributed to our study with his wide expertise in molecular diagnostics.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2021.107916.

References

Adrian-Kalchhauser, I., Burkhardt-Holm, P., Xu, P., 2016. An eDNA assay to monitor a globally invasive fish species from flowing freshwater. PLoS One 11 (1), e0147558. https://doi.org/10.1371/journal.pone.0147558.

Arabi, J., Judson, M.L.I., Deharveng, L., Lourenço, W.R., Cruaud, C., Hassanin, A., 2012. Nucleotide composition of CO1 sequences in Chelicerata (Arthropoda): detecting new mitogenomic rearrangements. J. Mol. Evol. 74 (1-2), 81–95. https://doi.org/10.1007/s00239-012-9490-7.

Bartsch, I., Davids, C., Deichsel, R., Di Sapatino, A., Gabrys, G., Gerecke, R., Gledhill, T., Jäger, P., Makol, J., Smit, H., van der Hammen, H., Weigmann, G., Wohltmann, A., Wurst, E., 2007. Chelicerata: Araneae, Acari I. In: Gerecke, R. (Ed.), Süsswasserfauna von Mitteleuropa. Spektrum Akademischer Verlag, München, Heidelberg, Neckar.

Beng, K.C., Corlett, R.T., 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiv. Conserv. Springer Netherlands. 29 (7), 2089–2121. https://doi.org/10.1007/s10531-020-01980-0.

Beniston, M., 2006. Mountain weather and climate: A general overview and a focus on climatic change in the Alps. Hydrobiologia 562 (1), 3–16. https://doi.org/10.1007/s10750-005-1802-0.

Bergsten, J., Bilton, D.T., Fujisawa, T., Elliott, M., Monaghan, M.T., Balke, M., Hendrich, L., Geijer, J., Herrmann, J., Foster, G.N., Ribera, I., Nilsson, A.N., Barraclough, T.G., Vogler, A.P., 2012. The effect of geographical scale of sampling on DNA barcoding. Syst. Biol. 61 (5), 851–869. https://doi.org/10.1093/sysbio/sys037.

Blackman, R., Ling, K.K.S., Harper, L., Shum, P., Hänfling, B., Handley, L.L., 2020. Targeted and passive environmental DNA approaches outperform established methods for detection of quagga mussels, Dreissena rostriformis bugensis in flowing water. Ecol. Evol. https://doi.org/10.1101/2020.01.07.890731.

Blackman, R.C., Mächler, E., Altermatt, F., Arnold, A., Beja, P., Boets, P., Egeter, B., Elbrecht, V., Filipe, A.F., Iwan Jones, J., Macher, J., Majaneva, M., Martins, F.M.S., Múrria, C., Meissner, K., Pawlowski, J., Schmidt Yáñez, P.L., Zizka, V.M.A., Leese, F., Price, B., Deiner, K., 2019. Advancing the use of molecular methods for routine freshwater macroinvertebrate biomonitoring - The need for calibration experiments. Metabarcod. Metagenom. 3, 49–57. https://doi.org/10.3897/mbmg.3.34735.

Blattner, L., Gerecke, R., Von Fumetti, S., 2019. Hidden biodiversity revealed by integrated morphology and genetic species delimitation of spring dwelling water mite species (Acari, Parasitengona: Hydrachnidia). Paras. Vectors 12, 1–13. https://doi.org/10.1186/s13071-019-3750-y.

Bossley, J.P., Smiley, P.C., 2019. Impact of student-induced disturbance on stream macroinvertebrates differs among habitat types. Sci. Rep. 9, 1–15. https://doi.org/10.1038/s41598-018-38210-1.

Boumans, L., Hogner, S., Brittain, J., Johnsen, A., 2016. Ecological speciation by temporal isolation in a population of the stonefly *Leuctra hippopus* (Plecoptera, Leuctridae). Ecol. Evol. 7 (5), 1635–1649. https://doi.org/10.1002/ece3.2638.

Bush, A., Compson, Z.G., Monk, W.A., Porter, T.M., Steeves, R., Emilson, E., Gagne, N., Hajibabaei, M., Roy, M., Baird, D.J., 2019. Studying ecosystems with DNA metabarcoding: lessons from biomonitoring of aquatic macroinvertebrates. Front. Ecol. Evol. 7, 1–12. https://doi.org/10.3389/fevo.2019.00434.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797.

S.A. Bustin R. Mueller T. Nolan Parameters for Successful PCR Primer Design R. Biassoni A. Raso Quantitative Real-Time PCR: Methods and Protocols 2020 Springer New York, New York, NY 5 22 10.1007/978-1-4939-9833-3_2.

Cantonati, M., Füreder, L., Gerecke, R., Jüttner, I., Cox, E.J., 2012. Crenic habitats, hotspots for freshwater biodiversity conservation: toward an understanding of their ecology. Freshw. Sci. 31 (2), 463–480. https://doi.org/10.1899/11-111.1.

Cantonati, M., Gerecke, R., Bertuzzi, E., 2006. Springs of the alps – sensitive ecosystems to environmental change: from biodiversity assessments to long-term studies. Hydrobiologia 562 (1), 59–96. https://doi.org/10.1007/s10750-005-1806-9.

Cantonati, M., Segadelli, S., Spitale, D., Gabrieli, J., Gerecke, R., Angeli, N., De Nardo, M. T., Ogata, K., Wehr, J.D., 2020. Geological and hydrochemical prerequisites of unexpectedly high biodiversity in spring ecosystems at the landscape level. Sci. Total Environ. 740, 140157. https://doi.org/10.1016/j.scitotenv.2020.140157.

Conte, J., Potoczniak, M.J., Tobe, S.S., 2018. Using synthetic oligonucleotides as standards in probe-based qPCR. Biotechniques 64 (4), 177–179. https://doi.org/10.2144/btn-2018-2000.

Currier, C.A., Morris, T.J., Wilson, C.C., Freeland, J.R., 2018. Validation of environmental DNA (eDNA) as a detection tool for at-risk freshwater pearly mussel species (Bivalvia: Unionidae). Aquat. Conserv. Mar. Freshw. Ecosyst. 28 (3), 545–558. https://doi.org/10.1002/aqc.v28.310.1002/aqc.2869.

Deiner, K., Altermatt, F., Gilbert, T., 2014. Transport distance of invertebrate environmental DNA in a natural river. PLoS One 9 (2), e88786. https://doi.org/10.1371/journal.pone.0088786.

Deiner, K., Knapp, R.A., Boiano, D.M., May, B., 2013. Increased accuracy of species lists developed for alpine lakes using morphology and cytochrome oxidase I for identification of specimens. Mol. Ecol. Resour. 13 (5), 820–831. https://doi.org/10.1111/men.2013.13.issue-510.1111/1755-0998.12130.

Deiner, K., Walser, J.C., Mächler, E., Altermatt, F., 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. Biol. Conserv. 183, 53–63. https://doi.org/10.1016/j.biocon.2014.11.018.

Di Sabatino, A., Cicolani, B., Gerecke, R., 2003. Biodiversity and distribution of water mites (Acari, Hydrachnidia) in spring habitats. Freshw. Biol. 48, 2163–2173. https://doi.org/10.1046/j.1365-2427.2003.01151.x.

Di Sabatino, A., Gerecke, R., Gledhill, T., Smit, H., 2010. Chelicerata: Acari II. In: Gerecke, R. (Ed.), Süsswasserfauna von Mitteleuropa. Spektrum Akademischer Verlag, Heidelberg, p. 234.

Eisenhauer, N., Bonn, A., Guerra, A., C.,, 2019. Recognizing the quiet extinction of invertebrates. Nat. Commun. 10, 1–3. https://doi.org/10.1038/s41467-018-07916-1.

Elbrecht, Vasco, Leese, Florian, 2015. Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. PLoS One 10 (7), e0130324. https://doi.org/10.1371/journal.pone.0130324.

Elbrecht, Vasco, Vamos, Ecaterina Edith, Meissner, Kristian, Aroviita, Jukka, Leese, Florian, Yu, Douglas, 2017. Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. Methods Ecol. Evol. 8 (10), 1265–1275. https://doi.org/10.1111/mee3.2017.8.issue-1010.1111/2041-210X.12789.

European Comission, 2000. The EU Water Framework Directive—integrated river basin management for Europe, Official Journal of the European Communities (OJ L 327).

Evans, Joshua R., Vis, Morgan L., 2020. Relative expression analysis of light-harvesting genes in the freshwater alga *Lympha mucosa* (Batrachospermales, Rhodophyta). J. Phycol. 56 (2), 540–548. https://doi.org/10.1111/jpy.v56.210.1111/jpy.12967.

Fait, Pauline, Demierre, Eliane, Ilg, Christiane, Oertli, Beat, 2020. Small mountain reservoirs in the Alps: New habitats for alpine freshwater biodiversity? Aquat. Conserv. Mar. Freshw. Ecosyst. 30 (4), 617–630. https://doi.org/10.1002/aqc.v30.410.1002/aqc.3306.

Fanin, Nicolas, Gundale, Michael J., Farrell, Mark, Ciobanu, Marcel, Baldock, Jeff A., Nilsson, Marie-Charlotte, Kardol, Paul, Wardle, David A., 2018. Consistent effects of

biodiversity loss on multifunctionality across contrasting ecosystems. Nat. Ecol. Evol. 2 (2), 269–278. https://doi.org/10.1038/s41559-017-0415-0.
Gerecke, R., Cantonati, M., Spitale, D., Stur, E., Wiedenbrug, S., 2011. The challenges of long-term ecological research in springs in the northern and southern Alps: Indicator groups, habitat diversity, and medium-term change. J. Limnol. 70, 168–187. https://doi.org/10.3274/JL11-70-S1-13.
Gerecke, Reinhard, Gledhill, Terence, Pešić, Vladimir, Smit, Harry (Eds.), 2016. Süßwasserfauna von Mitteleuropa, Bd. 7/2-3 Chelicerata. Springer Berlin Heidelberg, Berlin, Heidelberg.
Gerecke, Reinhard, Lehmann, Erwin O., 2005. Towards a longterm monitoring of Central European water mite faunas (Acari: Hydrachnidia and Halacaridae) - Considerations on the background of data from 1900 to 2000. Limnologica 35 (1-2), 45–51. https://doi.org/10.1016/j.limno.2004.09.001.
Gerecke, Reinhard, Martin, Peter, Gledhill, Terence, 2018. Water mites (Acari Parasitengona: Hydrachnidia) as inhabitants of groundwater-influenced habitats - considerations following an update of Limnofauna Europaea. Limnologica 69, 81–93. https://doi.org/10.1016/j.limno.2017.11.008.
Gieswein, A., Hering, D., Lorenz, A.W., 2019. Development and validation of a macroinvertebrate-based biomonitoring tool to assess fine sediment impact in small mountain streams. Sci. Total Environ. 652, 1290–1301. https://doi.org/10.1016/j.scitotenv.2018.10.180.
D.S. Glazier Reference Module in Earth Systems and Environmental Sciences 2014 Elsevier 10.1016/B978-0-12-409548-9.09322-2.
Gleason, J.E., Elbrecht, V., Braukmann, T.W.A., Hanner, R.H., Cottenie, K., 2020. Assessment of stream macroinvertebrate communities with eDNA is not congruent with tissue-based metabarcoding. Mol. Ecol. 1–13 https://doi.org/10.1111/mec.15597.
A. Gobiet S. Kotlarski A. Gobiet S. Kotlarski Future Climate Change in the European Alps Oxford Res. Encycl. Clim. Sci. 2020 1 23 https://doi.org/https://doi.org/10.1038/s41558-019-0676-5.
Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E., Taberlet, P., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol. Evol. 7, 1299–1307. https://doi.org/10.1111/2041-210X.12595.
Goldschmidt, Tom, 2016. Water mites (Acari, Hydrachnidia): powerful but widely neglected bioindicators – a review. Neotrop. Biodivers. 2 (1), 12–25. https://doi.org/10.1080/23766808.2016.1144359.
Lawson Handley, Lori, 2015. How will the "molecular revolution" contribute to biological recording? Biol. J. Linn. Soc. 115 (3), 750–766. https://doi.org/10.1111/bij.12516.
Harvey, Chris J., Fisher, Jennifer L., Samhouri, Jameal F., Williams, Gregory D., Francis, Tessa B., Jacobson, Kym C., deReynier, Yvonne L., Hunsicker, Mary E., Garfield, Newell, 2020. The importance of long-term ecological time series for integrated ecosystem assessment and ecosystem-based management. Prog. Oceanogr. 188, 102418. https://doi.org/10.1016/j.pocean.2020.102418.
Hering, Daniel, Meier, Carolin, Rawer-Jost, Claudia, Feld, Christian K., Biss, Regina, Zenker, Armin, Sundermann, Andrea, Lohse, Susanne, Böhmer, Jürgen, 2004. Assessing streams in Germany with benthic invertebrates: selection of candidate metrics. Limnologica 34 (4), 398–415. https://doi.org/10.1016/S0075-9511(04)80009-4.
Hernandez, Cecilia, Bougas, Bérénice, Perreault-Payette, Alysse, Simard, Anouk, Côté, Guillaume, Bernatchez, Louis, 2020. 60 specific eDNA qPCR assays to detect invasive, threatened, and exploited freshwater vertebrates and invertebrates in Eastern Canada. Environ. DNA 2 (3), 373–386. https://doi.org/10.1002/edn3.v2.310.1002/edn3.89.
Hotzy, R., Römheld, J., 2008. Aktionsprogramm Quellen in Bayern-Teil 2: Quellerfassung und–Bewertung. Bayer, Landesamt fur Umwelt, Augsbg.
Hunter, M.E., Ferrante, J.A., Meigs-Friend, G., Ulmer, A., 2019. Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. Sci. Rep. 9, 1–9. https://doi.org/10.1038/s41598-019-40977-w.
Jane, Stephen F., Wilcox, Taylor M., McKelvey, Kevin S., Young, Michael K., Schwartz, Michael K., Lowe, Winsor H., Letcher, Benjamin H., Whiteley, Andrew R., 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Mol. Ecol. Resour. 15 (1), 216–227. https://doi.org/10.1111/men.2014.15.issue-110.1111/1755-0998.12285.
Jonas Jourdan Robert B. O'Hara Roberta Bottarin Kaisa-Leena Huttunen Mathias Kuemmerlen Don Monteith Timo Muotka Dāvis Ozoliņš Riku Paavola Francesca Pilotto Gunta Springe Agnija Skuja Andrea Sundermann Jonathan D. Tonkin Peter Haase Effects of changing climate on European stream invertebrate communities: A long-term data analysis 621 2018 588 599 10.1016/j.scitotenv.2017.11.242.
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30 (4), 772–780. https://doi.org/10.1093/molbev/mst010.
Ketchum, H.R., Teel, C.J., Coates, O.F., Strey, M.T., Teel, P.D., Coates, C.J., Strey, O.F., Longnecker, A.M.T., 2009. Genetic variation in 12S and 16S mitochondrial rDNA genes of four geographically isolated populations of Gulf Coast Ticks (Acari: Ixodidae). longnecker Source. J. Med. Entomol. J. Med. Entomol 46, 482–489. https://doi.org/10.1603/033.046.0311.
Klymus, Katy E., Merkes, Christopher M., Allison, Michael J., Goldberg, Caren S., Helbing, Caren C., Hunter, Margaret E., Jackson, Craig A., Lance, Richard F., Mangan, Anna M., Monroe, Emy M., Piaggio, Antoinette J., Stokdyk, Joel P., Wilson, Chris C., Richter, Catherine A., 2020a. Reporting the limits of detection and quantification for environmental DNA assays. Environ. DNA 2 (3), 271–282. https://doi.org/10.1002/edn3.v2.310.1002/edn3.29.
Klymus, K.E., Ruiz Ramos, D.V., Thompson, N.L., Richter, C.A., 2020b. Development and testing of species-specific quantitative PCR assays for environmental DNA applications. J. Vis. Exp. 1–25 https://doi.org/10.3791/61825.
Kõressaar, T., Lepamets, M., Kaplinski, L., Raime, K., Andreson, R., Remm, M., 2018. Primer3-masker: Integrating masking of template sequence with primer design software. Bioinformatics 34, 1937–1938. https://doi.org/10.1093/bioinformatics/bty036.
Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23 (10), 1289–1291. https://doi.org/10.1093/bioinformatics/btm091.
C. Körner Alpine Plant Life 2nd ed. 2003 Springer-Verlag Berlin Heidelberg 10.1007/978-3-642-18970-8.
Krehenwinkel, H., Wolf, M., Lim, J.Y., Rominger, A.J., Simison, W.B., Gillespie, R.G., 2017. Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. Sci. Rep. 7, 1–12. https://doi.org/10.1038/s41598-017-17333-x.
Kuefner, Wolfgang, Ossyssek, Stefan, Geist, Juergen, Raeder, Uta, 2020. The silicification value: a novel diatom-based indicator to assess climate change in freshwater habitats. Diatom Res. 35 (1), 1–16. https://doi.org/10.1080/0269249X.2020.1722246.
Küry, Daniel, Lubini, Verena, Stucki, Pascal, 2016. Temperature patterns and factors governing thermal response in high elevation springs of the Swiss Central Alps. Hydrobiologia 793 (1), 185–197. https://doi.org/10.1007/s10750-016-2918-0.
Langone, M., Petta, L., Cellamare, C.M., Ferraris, M., Guzzinati, R., Mattioli, D., Sabia, G., 2020. SARS-CoV-2 in water services: presence and impacts. Environ. Pollut. 268, 115806. https://doi.org/10.1016/j.envpol.2020.115806.
Leese, Florian, Sander, Mandy, Buchner, Dominik, Elbrecht, Vasco, Haase, Peter, Zizka, Vera M.A., 2021. Improved freshwater macroinvertebrate detection from environmental DNA through minimized nontarget amplification. Environ. DNA 3 (1), 261–276. https://doi.org/10.1002/edn3.v3.110.1002/edn3.177.
Lencioni, Valeria, Bellamoli, Francesco, Paoli, Francesca, 2020. Multi-level effects of emerging contaminants on macroinvertebrates in Alpine streams: From DNA to the ecosystem. Ecol. Indic. 117, 106660. https://doi.org/10.1016/j.ecolind.2020.106660.
Liu, Mingxin, Clarke, Laurence J., Baker, Susan C., Jordan, Gregory J., Burridge, Christopher P., 2020. A practical guide to DNA metabarcoding for entomological ecologists. Ecol. Entomol. 45 (3), 373–385. https://doi.org/10.1111/een.v45.310.1111/een.12831.
Lor, Y., Schreier, T.M., Waller, D.L., Merkes, C.M., 2020. Using environmental DNA (eDNA) to detect the endangered Spectaclecase Mussel (*Margaritifera monodonta*). Freshw. Sci. 39, 837–847. https://doi.org/10.1086/71167.
Lubini, V., Knispel, S., Vinçon, G., 2012. Die Steinfliegen der Schweiz: Bestimmung und Verbreitung, in: Fauna Helvetica. CSCF&SEG, Neuchatel.
Lubini, V., Stucki, P., Vicentini, H., Küry, D., 2014. Ökologische Bewertung von Quell-Lebensräumen in der Schweiz. Entwurf für ein strukturelles und faunistisches Verfahren, Federal Office for the Environment (FOEN), Switzerland.
Mächler, Elvira, Deiner, Kristy, Spahn, Fabienne, Altermatt, Florian, 2016. Fishing in the water: effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. Environ. Sci. Technol. 50 (1), 305–312. https://doi.org/10.1021/acs.est.5b0418810.1021/acs.est.5b04188.s001.
Mächler, Elvira, Deiner, Kristy, Steinmann, Patrick, Altermatt, Florian, 2014. Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. Freshw. Sci. 33 (4), 1174–1183. https://doi.org/10.1086/678128.
Mastrotheodoros, Theodoros, Pappas, Christoforos, Molnar, Peter, Burlando, Paolo, Manoli, Gabriele, Parajka, Juraj, Rigon, Riccardo, Szeles, Borbala, Bottazzi, Michele, Hadjidoukas, Panagiotis, Fatichi, Simone, 2020. More green and less blue water in the Alps during warmer summers. Nat. Clim. Chang. 10 (2), 155–161. https://doi.org/10.1038/s41558-019-0676-5.
Mauvisseau, Q., Burian, A., Gibson, C., Brys, R., Ramsey, A., Sweet, M., 2019. Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. Sci. Rep. 9, 1–10. https://doi.org/10.1038/s41598-018-37001-y.
Meredith, C.S., Trebitz, A.S., Hoffman, J.C., 2019. Resolving taxonomic ambiguities: effects on rarity, projected richness, and indices in macroinvertebrate datasets. Ecol. Indic. 98, 137–148. https://doi.org/10.1016/j.ecolind.2018.10.047.
Messing, J., 1983. New M13 vectors for cloning. Methods in Enzymology. Elsevier 20–78.
Miccoli, F.P., Lombardo, P., Cicolani, B., 2013. Indicator value of lotic water mites (Acari: Hydrachnidia) and their use in macroinvertebrate-based indices for water quality assessment purposes. Knowl. Manag. Aquat. Ecosyst. (411), 08. https://doi.org/10.1051/kmae/2013075.
Morinière, Jérôme, Hendrich, Lars, Balke, Michael, Beermann, Arne J., König, Tobias, Hess, Monika, Koch, Stefan, Müller, Reinhard, Leese, Florian, Hebert, Paul D.N., Hausmann, Axel, Schubart, Christoph D., Haszprunar, Gerhard, 2017. A DNA barcode library for Germany's mayflies, stoneflies and caddisflies (Ephemeroptera, Plecoptera and Trichoptera). Mol. Ecol. Resour. 17 (6), 1293–1307. https://doi.org/10.1111/men.2017.17.issue-610.1111/1755-0998.12683.
Nadig, A., 1942. Hydrobiologische untersuchungen in quellen des schweizerischen nationalparkes im engadin:(unter besonderer berücksichtigung der insektenfauna). Sauerländer.
Oertli, Beat, Indermuehle, Nicola, Angélibert, Sandrine, Hinden, Hélène, Stoll, Aurélien, 2010. In: Pond Conservation in Europe. Springer Netherlands, Dordrecht, pp. 29–41. https://doi.org/10.1007/978-90-481-9088-1_4.
R. Pascual A. Nebra J. Gomà C. Pedrocchi O. Cadiach First data on the biological richness of Mediterranean springs Limnetica 39 2020 121 139 https://doi.org/10.23818/limn.39.09.

Piper, A.M., Batovska, J., Cogan, N.O.I., Weiss, J., Cunningham, J.P., Rodoni, B.C., Blacket, M.J., 2019. Prospects and challenges of implementing DNA metabarcoding for high-throughput insect surveillance. Gigascience 8, 1–22. https://doi.org/10.1093/gigascience/giz092.

Pozojević, I., Pešić, V., Goldschmidt, T., Gottstein, S., 2020. Crenal habitats: sources of water mite (Acari: Hydrachnidia) diversity. Diversity 12, 19–23. https://doi.org/10.3390/D12090316.

R Core Team R: A language and environment for statistical computing 2017.

Reiss, M., Martin, P., Gerecke, R., von Fumetti, S., 2016. Limno-ecological characteristics and distribution patterns of spring habitats and invertebrates from the Lowlands to the Alps. Environ. Earth Sci. 75, 1–11. https://doi.org/10.1007/s12665-016-5818-8.

Robinson, C.T., Oertli, B., 2009. Long-term biomonitoring of alpine waters in the Swiss National Park. Eco.mont 1, 23–34. https://doi.org/10.1553/eco.mont1s23.

Robinson, Christopher Thomas, Schmid, Dominik, Svoboda, Matthias, Bernasconi, Stefano M., 2008. Functional measures and food webs of high elevation springs in the Swiss alps. Aquat. Sci. 70 (4), 432–445. https://doi.org/10.1007/s00027-008-8125-y.

Rodríguez, A., Rodríguez, M., Córdoba, J.J., Andrade, M.J., 2015. Design of Primers and Probes for Quantitative Real-Time PCR Methods. In: Basu, C. (Ed.), PCR Primer Design. Springer, New York, New York, NY, pp. 31–56. https://doi.org/10.1007/978-1-4939-2365-6_3.

Rogora, M., Frate, L., Carranza, M.L., Freppaz, M., Stanisci, A., Bertani, I., Bottarin, R., Brambilla, A., Canullo, R., Carbognani, M., Cerrato, C., Chelli, S., Cremonese, E., Cutini, M., Di Musciano, M., Erschbamer, B., Godone, D., Iocchi, M., Isabellon, M., Magnani, A., Mazzola, L., Morra di Cella, U., Pauli, H., Petey, M., Petriccione, B., Porro, F., Psenner, R., Rossetti, G., Scotti, A., Sommaruga, R., Tappeiner, U., Theurillat, J.P., Tomaselli, M., Viglietti, D., Viterbi, R., Vittoz, P., Winkler, M., Matteucci, G., 2018. Assessment of climate change effects on mountain ecosystems through a cross-site analysis in the Alps and Apennines. Sci. Total Environ. 624, 1429–1442. https://doi.org/10.1016/j.scitotenv.2017.12.155.

Rosati, Melissa, Rossetti, Giampaolo, Cantonati, Marco, Pieri, Valentina, Roca, Josep R., Mesquita-Joanes, Francesc, 2017. Are aquatic assemblages from small water bodies more stochastic in dryer climates? An analysis of ostracod spring metacommunities. Hydrobiologia 793 (1), 199–212.

Rosset, V., Lehmann, A., Oertli, B., 2010. Warmer and richer? Predicting the impact of climate warming on species richness in small temperate waterbodies. Glob. Chang. Biol. 16, 2376–2387. https://doi.org/10.1111/j.1365-2486.2010.02206.x.

Sanches, Thiago M., Schreier, Andrea D., 2020. Optimizing an eDNA protocol for estuarine environments: Balancing sensitivity, cost and time. PLoS One 15 (5), e0233522. https://doi.org/10.1371/journal.pone.0233522.

Schabacker, Jenna C., Amish, Stephen J., Ellis, Bonnie K., Gardner, Beth, Miller, Dulaney L., Rutledge, Elizabeth A., Sepulveda, Adam J., Luikart, Gordon, 2020. Increased eDNA detection sensitivity using a novel high-volume water sampling method. Environ. DNA 2 (2), 244–251. https://doi.org/10.1002/edn3.v2.210.1002/edn3.63.

Senapati, Debabrata, Bhattacharya, Manojit, Kar, Avijit, Chini, Deep Sankar, Das, Basanta Kumar, Patra, Bidhan Chandra, 2019. Environmental DNA (eDNA): a promising biological survey tool for aquatic species detection. Proc. Zool. Soc. 72 (3), 211–228. https://doi.org/10.1007/s12595-018-0268-9.

Stadhouders, Ralph, Pas, Suzan D., Anber, Jeer, Voermans, Jolanda, Mes, Ted H.M., Schutten, Martin, 2010. The effect of primer-template mismatches on the detection and quantification of nucleic acids using the 5′ nuclease assay. J. Mol. Diagnostics 12 (1), 109–117. https://doi.org/10.2353/jmoldx.2010.090035.

Stein, Eric D., Martinez, Maria C., Stiles, Sara, Miller, Peter E., Zakharov, Evgeny V., Casiraghi, Maurizio, 2014. Is DNA barcoding actually cheaper and faster than traditional morphological methods: Results from a survey of freshwater bioassessment efforts in the United States? PLoS One 9 (4), e95525. https://doi.org/10.1371/journal.pone.0095525.

R. Stryjecki A. Bańkowska M. Gryzińska E. Sarnacka M. Rutkowska A. Zawal The use of molecular techniques in the taxonomy of water mites (Hydrachnidia, Acari) Acta Biol. 23 2016 23 110 https://doi.org/10.18276/ab.2016.23-10.

Stucki, P., Martinez, N., Roth, T., Küry, D., 2019. Methoden zur Untersuchung und Beurteilung von Fliessgewässern (IBCH_2019). Makrozoobenthos – Stufe F, in: Umwelt-Vollzug Nr. 1026. Federal Office for the Environment (FOEN), Switzerland, p. 59.

Sweeney, Bernard W., Battle, Juliann M., Jackson, John K., Dapkey, Tanya, 2011. Can DNA barcodes of stream macroinvertebrates improve descriptions of community structure and water quality? J. North Am. Benthol. Soc. 30 (1), 195–216. https://doi.org/10.1899/10-016.1.

Taylor, Sean C., Nadeau, Katia, Abbasi, Meysam, Lachance, Claude, Nguyen, Marie, Fenrich, Joshua, 2019. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. Trends Biotechnol. 37 (7), 761–774. https://doi.org/10.1016/j.tibtech.2018.12.002.

Thomas, Austen C., Tank, Samantha, Nguyen, Phong L., Ponce, Jake, Sinnesael, Mieke, Goldberg, Caren S., 2019. A system for rapid eDNA detection of aquatic invasive species. Environ. DNA 2 (3), 261–270. https://doi.org/10.1002/edn3.v2.310.1002/edn3.25.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L., Willerslev, E., 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Mol. Ecol. 21, 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x.

Thomsen, P.F., Willerslev, E., 2015. Environmental DNA - an emerging tool in conservation for monitoring past and present biodiversity. Biol. Conserv. 183, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019.

Toews, D.P.L., Brelsford, A., 2012. The biogeography of mitochondrial and nuclear discordance in animals. Mol. Ecol. 21, 3907–3930. https://doi.org/10.1111/j.1365-294X.2012.05664.x.

Tsuji, S., Iguchi, Y., Shibata, N., Teramura, I., Kitagawa, T., Yamanaka, H., 2018. Real-time multiplex PCR for simultaneous detection of multiple species from environmental DNA: an application on two Japanese Medaka species. Sci. Rep. 8, 1–5. https://doi.org/10.1038/s41598-018-27434-w.

Tsuji, Satsuki, Takahara, Teruhiko, Doi, Hideyuki, Shibata, Naoki, Yamanaka, Hiroki, 2019. The detection of aquatic macroorganisms using environmental DNA analysis—a review of methods for collection, extraction, and detection. Environ. DNA 1 (2), 99–108. https://doi.org/10.1002/edn3.2019.1.issue-210.1002/edn3.21.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S. G., 2012. Primer3-new capabilities and interfaces. Nucleic Acids Res. 40, 1–12. https://doi.org/10.1093/nar/gks596.

van der Loos, L.M., Nijland, R., 2020. Biases in bulk: DNA metabarcoding of marine communities and the methodology involved. Mol. Ecol. 1–19 https://doi.org/10.1111/mec.15592.

von Fumetti, Stefanie, Bieri-Wigger, Fabian, Nagel, Peter, 2017. Temperature variability and its influence on macroinvertebrate assemblages of alpine springs. Ecohydrology 10 (7), e1878. https://doi.org/10.1002/eco.v10.710.1002/eco.1878.

von Fumetti, Stefanie, Blattner, Lucas, 2016. Faunistic assemblages of natural springs in different areas in the Swiss National Park: a small-scale comparison. Hydrobiologia 793 (1), 175–184. https://doi.org/10.1007/s10750-016-2788-5.

von Fumetti, S., Felder, S., 2014. Faunistic Characterization of Alpine springs in the Swiss National Park. eco. mont-Journal Prot. Mt. Areas Res. 6, 43–49.

Waringer, J., Graf, W., 2011. Atlas of Central European Trichoptera Larvae.

Watts, Corinne, Dopheide, Andrew, Holdaway, Robert, Davis, Carina, Wood, Jamie, Thornburrow, Danny, Dickie, Ian A, 2019. DNA metabarcoding as a tool for invertebrate community monitoring: a case study comparison with conventional techniques. Austral Entomol. 58 (3), 675–686. https://doi.org/10.1111/aen.v58.310.1111/aen.12384.

Hannah Weigand Arne J. Beermann Fedor Čiampor Filipe O. Costa Zoltán Csabai Sofia Duarte Matthias F. Geiger Michał Grabowski Frédéric Rimet Björn Rulik Malin Strand Nikolaus Szucsich Alexander M. Weigand Endre Willassen Sofia A. Wyler Agnès Bouchez Angel Borja Zuzana Čiamporová-Zaťovičová Sónia Ferreira Klaas-Douwe B. Dijkstra Ursula Eisendle Jörg Freyhof Piotr Gadawski Wolfram Graf Arne Haegerbaeumer Berry B. van der Hoorn Bella Japoshvili Lujza Keresztes Emre Keskin Florian Leese Jan N. Macher Tomasz Mamos Guy Paz Vladimir Pešić Daniela Maric Pfannkuchen Martin Andreas Pfannkuchen Benjamin W. Price Buki Rinkevich Marcos A.L. Teixeira Gábor Várbíró Torbjørn Ekrem DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work 678 2019 499 524 10.1016/j.scitotenv.2019.04.247.

Weiss, M., Leese, F., 2016. Widely distributed and regionally isolated! Drivers of genetic structure in Gammarus fossarum in a human-impacted landscape. BMC Evol. Biol. 16, 153. https://doi.org/10.1186/s12862-016-0723-z.

Więcek, M., Martin, P., Lipinski, A., 2013. Water mites as potential long-term bioindicators in formerly drained and rewetted raised bogs. Ecol. Indic. 34, 332–335. https://doi.org/10.1016/j.ecolind.2013.05.019.

Wilcox, Taylor M., McKelvey, Kevin S., Young, Michael K., Jane, Stephen F., Lowe, Winsor H., Whiteley, Andrew R., Schwartz, Michael K., Willson, Richard C., 2013. Robust detection of rare species using environmental DNA: the importance of primer specificity. PLoS One 8 (3), e59520. https://doi.org/10.1371/journal.pone.0059520.

Williams, Kelly E., Huyvaert, Kathryn P., Piaggio, Antoinette J., Doi, Hideyuki, 2017. Clearing muddied waters: Capture of environmental DNA from turbid waters. PLoS One 12 (7), e0179282. https://doi.org/10.1371/journal.pone.0179282.

Wong, M.K.S., Nakao, M., Hyodo, S., 2020. Field application of an improved protocol for environmental DNA extraction, purification, and measurement using Sterivex filter. Sci. Rep. 10, 1–13. https://doi.org/10.1038/s41598-020-77304-7.

Wood, Susanna A., Biessy, Laura, Latchford, Janie L., Zaiko, Anastasija, von Ammon, Ulla, Audrezet, François, Cristescu, Melania E., Pochon, Xavier, 2020. Release and degradation of environmental DNA and RNA in a marine system. Sci. Total Environ. 704, 135314. https://doi.org/10.1016/j.scitotenv.2019.135314.

Zhang, Qi-Lin, Li, Hong-Wei, Dong, Zhi-Xiang, Yang, Xiao-Jie, Lin, Lian-Bing, Chen, Jun-Yuan, Yuan, Ming-Long, 2020. Comparative transcriptomic analysis of fireflies (Coleoptera: Lampyridae) to explore the molecular adaptations to fresh water. Mol. Ecol. 29 (14), 2676–2691. https://doi.org/10.1111/mec.v29.1410.1111/mec.15504.

Zinger, Lucie, Bonin, Aurélie, Alsos, Inger G., Bálint, Miklós, Bik, Holly, Boyer, Frédéric, Chariton, Anthony A., Creer, Simon, Coissac, Eric, Deagle, Bruce E., De Barba, Marta, Dickie, Ian A., Dumbrell, Alex J., Ficetola, Gentile Francesco, Fierer, Noah, Fumagalli, Luca, Gilbert, M. Thomas P., Jarman, Simon, Jumpponen, Ari, Kauserud, Håvard, Orlando, Ludovic, Pansu, Johan, Pawlowski, Jan, Tedersoo, Leho, Thomsen, Philip Francis, Willerslev, Eske, Taberlet, Pierre, 2019. DNA metabarcoding — Need for robust experimental designs to draw sound ecological conclusions. Mol. Ecol. 28 (8), 1857–1862. https://doi.org/10.1111/mec.2019.28.issue-810.1111/mec.15060.

Zizka, Vera M.A., Geiger, Matthias F., Leese, Florian, 2020. DNA metabarcoding of stream invertebrates reveals spatio-temporal variation but consistent status class assessments in a natural and urban river. Ecol. Indic. 115, 106383. https://doi.org/10.1016/j.ecolind.2020.106383.

Chapter V

General Discussion and Conclusion

Despite the scarcity of supporting empirical data, the assumption of a high degree of habitat isolation among springs has been one of the main arguments to consider springs as vulnerable ecosystems and promote their importance for landscape management and nature conservation (Cantonati et al., 2012; Davis et al., 2017). Therefore, one primary aim of this thesis was to provide conclusions on the degree of spring habitat interconnection by inferring the population genomic structure of a strictly crenobiontic species, the water mite *Partnunia steinmanni*. Compared to other taxa, water mites qualify for this purpose because of their high degree of spring habitat preference and evolutionary background that may have favoured speciation in springs (Dabert et al., 2010, 2016; Walter & Proctor, 2013; Gerecke et al., 2018). Furthermore, they exhibit rather weak dispersal abilities with migration limited to a phoretic phase during the parasitic larval stage (Di Sabatino et al., 2003; Walter & Proctor, 2013). Pioneer investigations on spring habitat isolation in Chapter III were therefore conducted on a system likely to reveal putative population isolation, an ideal baseline to further research spring interconnection including more actively dispersing species.

Water mites, however, are rather understudied and molecular methods as well as basic evolutionary insights into that taxon need to be developed first to enable more advanced investigations. Therefore, I assessed the mainly morphology-based species description and delimitation with molecular genetic methods, re-evaluated basic aspects of water mite taxonomy by phylogenetic placement and developed a genetic species identification database for the most abundant European spring water mite species (Chapter II). Results showed good overall congruence among genetic vs. traditional methods and fully supported the initially assumed taxonomical classification. In congruence with the phylogenetic inferences conducted

by Dabert et al. (2016), results strongly support the monophyly of Hydrachnidia with a new broad empirical data set. Additionally, the Hydryphantoidea are shown to be monophyletic and its members presumably share a common ancestor, which was questioned until recently (Dabert et al., 2016). Supplementary to these findings, the phylogenetic and distance-based clustering of specimens revealed hidden genetic diversity, indicating species putatively new to science and an underestimation of spring water mite diversity. The final description and definition of the newly discovered genotypes as distinct species, however, needs extended sampling, thorough morphological examination as well as an assessment of the species-specific autecology and contact zones to separate high intraspecific divergence from speciation processes in each particular case (see e.g., discussions in Harvey et al., 2019).

Among populations morphologically attributed to *Partnunia steinmanni* sensu Di Sabatino et al. (2010), two different genotypes (here called A and B) could be identified. The investigated specimens belonging to *P.* cf. *steinmanni* B showed a rather northern distribution, and genotype A was dominant across the Alps in populations located in the Swiss National Park, the Adamello-Brenta Nature Park and around Zermatt in Switzerland. Furthermore, clade A showed to be more closely related to the crenophilous/rhithrobiontic *Partnunia angusta* (Koenike, 1893) that exhibits a manly alpine distribution area (Di Sabatino et al., 2003, 2010), indicating potentially two reproductively isolated species, an alpine (= genotype A) and a northern species (= genotype B). *Partnunia steinmanni* not only exhibits high genetic diversity, alpine distribution and high degree of spring restriction, but also belongs to Hydryphantoidea, a monophyletic water mite superfamily that is known to be phylogenetically basal and therefore can be assumed to represent a broad time scale of evolutionary events (Dabert et al., 2016; Blattner et al., 2019 and references therein). *Partnunia steinmanni* populations across the Alps were then sampled, and the population genomic structure was investigated (Chapter III) to contribute to the understanding of alpine springs as insular habitats (Von Fumetti & Blattner,

2017). The strong genetic distinctiveness that was revealed, finally showed a high degree of population isolation and therefore isolation of springs, presumably over a relatively long period of time. With these results, I was able to support the original presumption of the island-like character of springs. Furthermore, my results revealed an underestimation of species richness and also showed that springs can exhibit a high degree of genetic distinctiveness, which is the basis of reproductive isolation and therefore enables further speciation over time (Harvey et al., 2019; Rader et al., 2019).

Increasing anthropogenic influence on springs can be expected and as shown in Chapter I, direct impact such as decreasing spring discharge due to groundwater overuse as well as indirect, human-induced climate change effects threaten natural spring ecosystems. To effectively monitor potential species loss and consequently decreasing environmental integrity of spring biodiversity hotspots (see Chapter II and III), I developed a set of qPCR assays to detect alpine spring-dwelling species in eDNA water samples (Chapter IV; Blattner et al., 2021). The detection of bioindicator species in eDNA samples circumvents many substantial drawbacks of conventional methods (Elbrecht et al., 2017; Blackman et al., 2019) and the non-invasive and cost-effective character of an eDNA qPCR approach can be seen as highly beneficial, especially when applied in protected areas such as National Parks. With the developed method, specifically the chosen Hydrachnidia, Trichoptera, and Plecoptera species can be detected. Such an approach, however, is not suitable to assess and monitor overall biodiversity and for that purpose needs to be supplemented with traditional as well as eDNA metabarcoding techniques (e.g., Taberlet et al., 2012; Leese et al., 2021). Despite a gradual growth of available sequence data, one major challenge would be developing reliable genetic species identification databases (Thomsen & Willerslev, 2015; Weigand et al., 2019; Beng & Corlett, 2020) for alpine spring species. In addition to the water mite identification sequences provided (Chapter II), many other taxa still lack dependable references.

In conclusion, I provided novel insight into the genetic species delimitation, identification, phylogeny and diversity of spring-dwelling water mite species. For the first time, I empirically showed that alpine springs are strongly isolated biodiversity islands with a high potential for ongoing speciation processes. Furthermore, I developed new eDNA spring bioindication assays to non-invasively monitor and assess ecosystems integrity. In addition to the conceptual contributions to crenobiology, the different studies conducted in this doctoral thesis also provide empirical data and new methodological approaches to advance further acorological research, a scientific discipline that is underestimated although having high potential to shed light on complex interactions between various environments, ecosystems, and its biota.

As presented in the general introduction, freshwater spring ecosystems and the species characteristic for these habitats show a relatively long history of scientific research and have been studied intensively. Despite this substantial base of knowledge and the data as well as conclusions that I was able to contribute, many open research questions, such as the evolutionary context of spring adaptation and specialization, still remain unanswered. Main challenges are, among others, the complexity of interactions among the diverse spring species and environmental conditions. The concept of "Ecohydrogeology" proposed by Cantonati et al. (2020) and defined as "*The unifying, synthetic scientific field integrating the approaches of ecology and hydrogeology in the study of groundwater-related ecosystems, habitats, organisms, and socio- cultural processes to advance understanding, stewardship, and policy*", describes the different spheres that need to be taken into account to holistically understand springs. As shown in this Ph.D. thesis, each of these entities, however, needs to be studied separately to provide the knowledge necessary to advance crenobiology.

References

Beng, K. C., & R. T. Corlett, 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation* Springer Netherlands 29: 2089–2121, https://doi.org/10.1007/s10531-020-01980-0.

Blackman, R. C., E. Mächler, F. Altermatt, A. Arnold, P. Beja, P. Boets, B. Egeter, V. Elbrecht, A. F. Filipe, J. Iwan Jones, J. Macher, M. Majaneva, F. M. S. Martins, C. Múrria, K. Meissner, J. Pawlowski, P. L. Schmidt Yáñez, V. M. A. Zizka, F. Leese, B. Price, & K. Deiner, 2019. Advancing the use of molecular methods for routine freshwater macroinvertebrate biomonitoring - The need for calibration experiments. *Metabarcoding and Metagenomics* 3: 49–57, https://doi.org/10.3897/mbmg.3.34735.

Blattner, L., J. N. Ebner, J. Zopfi, & S. von Fumetti, 2021. Targeted non-invasive bioindicator species detection in eDNA water samples to assess and monitor the integrity of vulnerable alpine freshwater environments. *Ecological Indicators* Elsevier Ltd 129: 107916, https://doi.org/10.1016/j.ecolind.2021.107916.

Blattner, L., R. Gerecke, & S. Von Fumetti, 2019. Hidden biodiversity revealed by integrated morphology and genetic species delimitation of spring dwelling water mite species (Acari, Parasitengona: Hydrachnidia). *Parasites and Vectors* BioMed Central 12: 1–13, https://doi.org/10.1186/s13071-019-3750-y.

Cantonati, M., L. Füreder, R. Gerecke, I. Jüttner, & E. J. Cox, 2012. Crenic habitats, hotspots for freshwater biodiversity conservation: toward an understanding of their ecology. *Freshwater Science* 31: 463–480, https://doi.org/10.1899/11-111.1.

Cantonati, M., L. E. Stevens, S. Segadelli, A. E. Springer, N. Goldscheider, F. Celico, M. Filippini, K. Ogata, & A. Gargini, 2020. Ecohydrogeology: The interdisciplinary convergence needed to improve the study and stewardship of springs and other groundwater-dependent habitats, biota, and ecosystems. *Ecological Indicators* Elsevier 110: 105803, https://doi.org/10.1016/j.ecolind.2019.105803.

Dabert, M., H. Proctor, & J. Dabert, 2016. Higher-level molecular phylogeny of the water mites (Acariformes: Prostigmata: Parasitengonina: Hydrachnidiae). *Mol. Phylo. and Evol.* 101: 75–90,https://doi.org/10.1016/j.ympev.2016.05.004

Dabert, M., W. Witalinski, A. Kazmierski, Z. Olszanowski, & J. Dabert, 2010. Molecular phylogeny of acariform mites (Acari, Arachnida): Strong conflict between phylogenetic signal and long-branch attraction artifacts. *Molecular Phylogenetics and Evolution* Elsevier Inc. 56: 222–241, https://doi.org/10.1016/j.ympev.2009.12.020.

Davis, J. A., A. Kerezsy, & S. Nicol, 2017. Springs: Conserving perennial water is critical in arid landscapes. *Biological Conservation* Elsevier Ltd 211: 30–35, https://doi.org/10.1016/j.biocon.2016.12.036.

Di Sabatino, A., B. Cicolani, & R. Gerecke, 2003. Biodiversity and distribution of water mites (Acari, Hydrachnidia) in spring habitats. *Freshwater Biology* 48: 2163–2173, https://doi.org/10.1046/j.1365-2427.2003.01151.x.

Di Sabatino, A., R. Gerecke, T. Gledhill, & H. Smit, 2010. Chelicerata: Acari II In Gerecke, R. (ed), Süsswasserfauna von Mitteleuropa. Spektrum Akademischer Verlag, Heidelberg: 234.

Elbrecht, V., E. E. Vamos, K. Meissner, J. Aroviita, & F. Leese, 2017. Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. *Methods in Ecology and Evolution* 8: 1265–1275, https://doi.org/10.1111/2041-210X.12789.

Gerecke, R., P. Martin, & T. Gledhill, 2018. Water mites (Acari Parasitengona: Hydrachnidia) as inhabitants of groundwater-influenced habitats - considerations following an update of Limnofauna Europaea. *Limnologica* , https://doi.org/10.1016/j.limno.2017.11.008.

Harvey, M. G., S. Singhal, & D. L. Rabosky, 2019. Beyond Reproductive Isolation: Demographic Controls on the Speciation Process. *Annual Review of Ecology, Evolution, and Systematics* 50: 75–95, https://doi.org/10.1146/annurev-ecolsys-110218-024701.

Leese, F., M. Sander, D. Buchner, V. Elbrecht, P. Haase, & V. M. A. Zizka, 2021. Improved freshwater macroinvertebrate detection from environmental DNA through minimized nontarget amplification. *Environmental DNA* 3: 261–276, https://doi.org/10.1002/edn3.177.

Rader, R. B., P. J. Unmack, W. F. Christensen, & X. Jiang, 2019. Connectivity of two species with contrasting dispersal abilities: A test of the isolated tributary hypothesis. *Freshwater Science* 38: 142–155, https://doi.org/10.1086/701671.

Taberlet, P., E. Coissac, M. Hajibabaei, & L. H. Rieseberg, 2012. Environmental DNA. *Molecular Ecology* 21: 1789–1793, https://doi.org/10.1111/j.1365294X.2012.05542

Thomsen, P. F., & E. Willerslev, 2015. Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation* Elsevier Ltd 183: 4–18, https://doi.org/10.1016/j.biocon.2014.11.019.

Von Fumetti, S., & L. Blattner, 2017. Faunistic assemblages of natural springs in different areas in the Swiss National Park: a small-scale comparison. *Hydrobiologia* Springer International Publishing 793: 175–184, https://doi.org/10.1007/s10750-016-2788-5.

Walter, D. E., & H. C. Proctor, 2013. Mites : Ecology , Evolution & Behaviour. Springer-Verlag, Dodrecht.

Weigand, H., A. J. Beermann, F. Čiampor, F. O. Costa, Z. Csabai, S. Duarte, M. F. Geiger, M. Grabowski, F. Rimet, B. Rulik, M. Strand, N. Szucsich, A. M. Weigand, E. Willassen, S. A. Wyler, A. Bouchez, A. Borja, Z. Čiamporová-Zaťovičová, S. Ferreira, K. D. B. Dijkstra, U. Eisendle, J. Freyhof, P. Gadawski, W. Graf, A. Haegerbaeumer, B. B. van der Hoorn, B. Japoshvili, L. Keresztes, E. Keskin, F. Leese, J. N. Macher, T. Mamos, G. Paz, V. Pešić, D. M. Pfannkuchen, M. A. Pfannkuchen, B. W. Price, B. Rinkevich, M. A. L. Teixeira, G. Várbíró, & T. Ekrem, 2019. DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. Science of the Total Environment. Elsevier B.V., 499–524, https://doi.org/10.1016/j.scitotenv.2019.04.247.

Acknowledgements

Many people have assisted me during my doctoral thesis. Although I am grateful to everyone who supported me, I would like to point out a few essential persons and institutions that made my work possible.

First of all, I would like to thank PD Dr. Stefanie von Fumetti for her support and mentorship at any stage of my Ph.D. thesis. Among many things, I appreciate her trust in me and the freedom that I had to investigate my own research questions. I also want to thank her for the possibility to experience the academic world holistically, and among other things, participate in conferences, supervise students, build own collaborations, be involved in teaching, have insight into the process of a research grant application, and even be responsible for infrastructure.

Furthermore, I want to thank Prof. Dr. Moritz Lehmann for co-supervising my Ph.D. thesis and PD Dr. Hans Jürgen Hahn for providing his expertise.

Then, I want to particularly point out the assistance that I received from Prof. Dr. Reinhard Gerecke. The numerous discussions we had and the work we did together tremendously enriched all aspects of my thesis. Without him, many projects would not have been possible.

Nathanael Beck and Joshua Ebner helped me during every stage of my thesis by providing scientific, technical, and conceptual knowledge, and supported me morally during challenging periods.

Prof. em. Dr. Peter Nagel and Prof. Dr. Oliver Heiri scientifically supported my work and provided the institutional background necessary.

I further closely collaborated with Dr. Jakob Zopfi, Dr. Kay Lucek, and PD Dr. Daniel Berner, who all provided valuable contribution to my projects.

Among many students and colleagues who helped me, I would like to thank Alicia Escher for her assistance during numerous field trips.

All of my studies were conducted in the Swiss National Park, the UNESCO Biosphera Engiadina Val Müstair, and other protected areas across the Alps that approved my work and provided fieldwork assistance.

Finally, the support of my family: Dr. Claudia Muralda, Peter Blattner, Florence Blattner, and my girlfriend Marlen Ursella, was essential for the success of this thesis. I would not be where I am today without them.