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

Lucas Blattner1 | Kay Lucek2 | Nathanael Beck1 | Daniel Berner3 | Stefanie von Fumetti1

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
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, Fürderer, 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., 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).

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

![Figure 1 Living Partnunia steinmanni specimen](image-url)
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

Particular 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 (Adamello-Brenta NP), including the Rhine (Mercantour NP and Rifelbord WA), Po (Adelma-Brenta NP), and the Danube (Swiss National Park (Swiss NP), Berchtesgaden NP, and Gesäuse NP) catchments. Additionally, we included three populations located in the highly protected Swiss National Park (Swiss NP) (Table 1). The northernmost site was sampled in the Berchtesgaden National Park (Berchtesgaden NP) 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 (Adamello-Brenta NP), including the Rhine, Po, and Danube catchments. Each 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 DNAAdvance Kit (Beckman Coulter Life Sciences, Indianapolis, USA). Whole mite individuals were processed in 96 deep well plates according to manufacturer protocol with a final elution volume of 50 µL. DNA was stored at −20°C until further processing. 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.
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 Kozarzewa & 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 H₂O₂ 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 1X 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 SPRSelect (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 (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 1X 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 1.8X SPRSelect (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 × 10⁹ reads (approximately 5.6 × 10⁸ 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 adapters, 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 preprocessed 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 and 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 <5x and >800x 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 172x 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 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 ($\delta_{FIS}$) 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 ($\delta_{M}$ = 0.1), allele frequencies ($\delta_{F}$ = 0.55), and inbreeding coefficients ($\delta_{F}$ = 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).
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 springs 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
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 $H_e$ ± SD = 0.113 ± 0.023;
mean $H_o \pm SD = 0.122 \pm 0.0273$) (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 $9.9 \pm 4.3$ (mean $\pm SD$) 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 $m \pm SD = 0.01 \pm 0.006$) and genetic exchange almost exclusively...
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 unpaired 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—genetic differentiation between populations from different springs) 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 & Janssen 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 (Galbraith & 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 Nₑ 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 Rifelsbord 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 degree of insularity of alpine spring habitats, likely shaped by sciCORE (http://scico.re.unibas.ch/) scientific computing centre and study area contains populations with a unique genetic make-up, dispersal abilities and low inter-population migration rates such as alpine headwater environments. Crenobiontic species with limited main catchment areas as previously shown to be characteristic for gle, well-defined species.

Given the strong population structure, a thorough re-evaluation 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). The genomic consequences of limited dispersal.

**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.

**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., Canu, K., Cavanaugh, M., Charowhas, C., Clark, K., Dondoshansky, I., Feolo, M., Fitzpatrick, L., Funk, K., Geer, L. Y., Gorelenkov, V., ... Zbic, 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., Jeffreys, 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., Wichelem, 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

**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
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 and parasitic clines. Proceedings of the National Academy of Sciences, 116(2), 525–541. https://doi.org/10.1073/pnas.1821056116

Kozarewa, I., & Turner, D. J. (2011). 96-plex molecular barcoding for the Illumina genome analyzer. In Y. M. Kwon, & S. C. Ricke (Eds.), Aquatic eukaryotic algae: perspectives for the analysis of genetic data of diploids and polyploids. Molecular Ecology Resources, 2011, 1126–1131. https://doi.org/10.1111/j.1755-0998.2011.02939.x

Lai, G. G., Padedda, B. M., Ector, L., Wetzel, C. E., Lugliè, A., & Cantonati, M. (2020). Mediterranean karst springs: Diatom biodiversity, strontium enrichment. Journal of Limnology, 79(1), 185–197. https://doi.org/10.1007/s10750-016-0218-0

Lai, G. G., Paddeka, 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

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 terra-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. EMBO Journal, 17(1), 10–12. https://doi.org/10.1486/ems.177.200

Martin, P., Koester, M., Schynawa, L., & Gergs, R. (2015). First detection of prey DNA in Hydrobates 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 in 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:1017985137212

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

Nielsen, 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., Gömä, J., Pedrocchi, C., & Cadiach, O. (2020). First data on the biological richness of Mediterranean springs. Limnética, 39(1), 121–139. https://doi.org/10.23818/litm.39.09

Pauls, S. U., Lumbsch, H. T., & Haase, P. (2006). Phylogeography of the montane caddisfly Drusus discolor: Evidence for multiple refugia and
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. (2022). Intra-Alpine Islands: Population genomic inference reveals high degree of isolation between freshwater spring habitats. Diversity and Distributions, 28, 291–305. https://doi.org/10.1111/ddi.13461