Genetic diversity management of sculpin (Cottus spp.) and brown trout (Salmo trutta) in the Palatinate Forest-North Vosges Biosphere Reserve

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Abstract – Protected areas can make an important contribution to the conservation of genetic diversity in the current biodiversity crisis. We have examined two representative freshwater fish taxa, Cottus spp. and Salmo trutta, in 15 midmountain headwaters of the Franco-German Palatinate Forest-North Vosges Biosphere Reserve in Central Europe to facilitate freshwater genetic diversity protection. Population genetic analyses of microsatellites and mtDNA showed lower genetic diversity, but distinctly differentiated genetic structure in Cottus spp., and higher diversity, but less differentiated structure in Salmo trutta. Phylogenetic analyses of mtDNA designated most sculpin to Cottus gobio, but also identified the first known population of Cottus rhenanus in the region. In addition to species-specific recommendations, we derived stream-specific guidance in an attempt to make optimal use of the combined genetic information on both taxa for habitat-oriented management prioritization and improved conservation of freshwater genetic diversity.

Keywords: Biodiversity loss / salmonids / cottids / River Rhine / Alsace

1 Introduction

The present biodiversity crisis (Barnosky et al., 2011) includes global loss of genetic variation in wild populations (Leigh et al., 2019). Intraspecific genetic variation is a foundational component of biodiversity, promotes ecosystem structure and functioning, and supports nature’s contributions to people (e.g., Des Roches et al., 2021; Hoban et al., 2022). Nevertheless, information on genetic variation is still not sufficiently considered in current conservation policy and decision making but improved standardization of collecting, harmonizing, and interpreting genetic data can help to better operationalize interspecific variation for conservation and management (Hoban et al., 2022 and citations therein). Regional assessments can not only improve the usability of their findings for regional managers and authorities, but also contribute to enable large-scale analyses by using standardized fundamental metrics (Genetic Composition EBVs; Hoban et al., 2022).

Freshwater habitats are biodiversity hotspots (Strayer and Dudgeon, 2010), but their current loss of species is disproportionately higher than in marine or terrestrial ecosystems (Tickner et al., 2020) and many European freshwater fish species show negative population trends (e.g., Deinet et al., 2020). Protection and restoration of critical freshwater habitats has been suggested as a priority action to “bend the curve of freshwater biodiversity loss” (Tickner et al., 2020). Yet, international efforts to conserve and facilitate the sustainable use of biodiversity currently require more actions and goals specific to the conservation of freshwater ecosystems and their species, genetic and functional diversity (Darwall et al., 2018).

Biosphere reserves and the UNESCO Man and Biodiversity (MAB) program aim to enable the protection and, at the same time, the sustainable use of biodiversity, and many reserves include valuable freshwater habitats. Conservation of biodiversity is one main function of biosphere reserves and this explicitly includes genetic variation particularly for their core zones. Activities in surrounding buffer zones should support scientific research and monitoring. To support the implementation of these goals, we have studied the genetic diversity and population structure of characteristic fish species in headwaters of a Central European biosphere reserve and propose a habitat-centered approach to aid in the development of habitat management measures to conserve genetic diversity.

The Palatinate Forest-North Vosges Biosphere Reserve at the French-German border extends broadly over a densely forested low mountain range. The landscape is characterized as a biodiversity hotspot and adjacent to the Rhine river. This is an Open Access article distributed under the terms of the Creative Commons Attribution License CC-BY-ND (https://creativecommons.org/licenses/by-nd/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. If you remix, transform, or build upon the material, you may not distribute the modified material.
by numerous fine substrate dominated brooks and streams. The freshwater sculpin bullhead (*Cottus gobio*) and brown trout (*Salmo trutta*) are, as in many other western and central European low mountain areas, typical fish species of the region and both are listed as index species of the biosphere reserve (Simon, 2003).

Sculpin and brown trout often occur syntopically, but differ in many ecological traits relevant for appropriate conservation and management measures. For instance, brown trout have better swimming capacities than the benthic, swim bladderless sculpin (e.g., Taugbol et al., 2019; Egger et al., 2021). Thus, their dispersal abilities differ and gene flow between sculpin populations may be affected more strongly by small river barriers (e.g., culverts, ramps) than between brown trout populations (e.g. Junker et al., 2012; cp. Hale et al., 2016). Further, in contrast to sculpin, brown trout are highly appreciated for recreational fisheries and hence often intensively managed. Finally, in western and central Europe these species are often the only ray finned fish species in small headwaters. Therefore, the combined study of these two species in particular with high resolution microsatellite markers is a promising approach to help managers make decisions about conserving genetic diversity from a habitat-based perspective, rather than just from the perspective of a single species.

In Europe there are currently 15 species of freshwater sculpin in the genus *Cottus* that are recognized (Freyhof et al., 2005). Three of these species occur in the broader study area in the Upper Rhine region, namely *C. gobio*, *C. perifretum* and *C. rhenanus* (Freyhof and Kottelat, 2008a, 2008b; Freyhof, 2011). *Cottus perifretum* and *C. rhenanus* were first described in the taxonomic revision of European *Cottus* species by Freyhof et al. (2005). To our knowledge, no detailed distribution mapping of the three species has taken place in the Upper Rhine tributaries since then. Thus, basic data for appropriate management, even at the species level, is lacking. Furthermore, morphological characters of *C. gobio* and *C. rhenanus* are highly variable (Freyhof et al., 2005), so that species identification in the field may be very difficult or even impossible, especially for practitioners. This problem can further contribute to the lack of species distribution data. However, all three relevant species correspond well to genetic clades or haplogroups found in earlier studies (Freyhof et al., 2000) by Englbrecht et al. (2000) and Volckaert et al. (2002). Thus, analyses of mitochondrial DNA (mtDNA) can enable species identification and help in overcoming knowledge gaps regarding the current distribution of sculpin species in the Palatinate Forest-North Vosges Biosphere Reserve.

To assist in improving the conservation and management of the genetic diversity in significant freshwater habitats in the Palatinate Forest-North Vosges Biosphere Reserve and to contribute to current global efforts to better integrate genetic diversity in conservation policies, we examined sculpin and brown trout by means of high-resolution microsatellite markers and mtDNA control region sequencing. For these purposes, we provide (1) a comprehensive population genetic characterization of these representative freshwater fish from 15 streams and (2) discuss possible implications of our findings for habitat-centered management and conservation in the reserve. Furthermore, mtDNA analyses revealed the presence of two different *Cottus* species in the reserve, which is additional and valuable information for conservation and management.

2 Materials and methods

2.1 Study sites

The Franco-German transboundary “Palatinate Forest-North Vosges” UNESCO Biosphere Reserve is located between the departments Bas Rhin and Moselle in north-east France and the federal state Rhineland-Palatinate in south-west Germany. The reserve covers an area of 3018 km². The landscape is characterized by densely forested low mountain ranges of Triassic Buntsandstein formations and the numerous small headwaters and rivers are predominantly fine substrate-rich, siliceous, and oligotrophic. In the streams there are numerous barriers, especially small ones, such as culverts and steps for bed stabilization, but also impoundments and weirs. Typically, slopes are between one and eight percent. We investigated 15 stream sites in five sub-catchments east of the main watershed in the reserve between rivers Moselle and Rhine, i.e., direct tributaries of the Rhine. All sites were in or at the border of buffer areas of the biosphere reserve, except one site (*Que*) in a transition area and one site (*Bab*) just outside the reserve (Fig. 1, Tab. 1).

Sites were selected to minimize the potential influence of fisheries management, i.e., all surveyed streams were so small that they are unlikely to be used for fishing, and no evidence of recent stocking was found for any of the waters before sampling. However, the full stocking history of the region and the selected sites ultimately remains unclear because we could not a priori rule out or assume undocumented stocking in the study areas or immigration of stocked fish. The population *Bab* outside of the reserve was included because until several years ago it served as a donor population for brown trout rearing for stocking efforts in nearby regions (e.g. Black Forest).

2.2 Sampling

In June 2019, and March 2021 (sites *Sub, Deb, Que*) backpack electrofishing was performed with partners (see acknowledgements) to acquire tissue samples. Fishes were anaesthetized, their species, or genus, and total length were documented, and fin clips were taken. Tissue samples were preserved in 96% ethanol on site and stored at 20°C in the lab.

2.3 Genetic analysis

Tissue was incubated with 5% Chelex 100 (Bio-Rad) and 0.06 mg Proteinase K (Qiagen) at 56°C for 2 h and 100°C for 30 min for DNA extraction. Nuclear DNA (n = 721) was then examined at ten microsatellite loci for sculpin (n = 255), and twelve loci for trout (n = 466) respectively, in six (multi load) multiplex-PCRs. Detailed locus and primer information (Tab. S1-1), PCR protocols (Tab. S1-2), and cycling parameters (Tab. S1-3) are provided in Supplemental Information S-1. At locus Cg01016 we found one allele (“136”) in one sculpin population (Bab) that appeared to fall out of the regular repeat length and was highly influential in
data analyses. Thus, we bidirectionally sequenced 3 homozygous samples with allele “136” and 4 homozygous samples with allele “137”, to rule out that this allele is an artefact (e.g., scoring error), as detailed in Tables S1-4 and S1-5.

The mitochondrial control region of 99 sculpin and 100 brown trout was amplified with primers PST (5’-CCCAAAGCTAAAATTCTAAAT-3’) and FST (5’-GCTTTAGTTAAGCTACGC-3’) (Cortey and Garcia-Marin, 2002) and bidirectionally sequenced (Tabs. S1-6 and S1-7). Sequencing was primarily done to identify sculpin species and we analyzed 10–12 specimens per site (except Ltb: 5). To allow further comparisons between sculpin and brown trout and complement microsatellite analyses, we additionally sequenced 10–12 brown trout from those 9 sites where both taxa were present (Tab. 1). Sequences (mtDNA and microsatellite Cgo1016) were edited and aligned using the software geneious prime (Biomatters Ltd.) and the Megablast implementation was used with mtDNA haplotypes to identify identical sequences deposited in GenBank and all haplotypes found in this study were submitted to GenBank (accession numbers OP186296–OP186308).

We determined the size range of all microsatellite alleles [base pairs (bp)] and the total number of alleles per locus and consequently rejected one locus (Cgo91) for sculpin and one locus (OMM1323) for brown trout because they were monomorphic (Tab. S1-1). To inspect data quality, occurrence of null alleles was estimated after Brookfield (1996) with the null.all function of the PopGenReport package v. 3.0.4 (Adamack and Gruber, 2014) using first the whole dataset per species and then the single populations per species in R v. 4.1.2 (R Core Team, 2021). Deviations from Hardy-Weinberg equilibrium (HWE) were tested per locus and population using the exact test of the hw.test function of the pegas v. 1.1 R package (Paradis, 2010) with 10,000 Monte-Carlo iterations.

2.4 Genetic diversity and inbreeding

To characterize genetic diversity, we calculated the means over microsatellite loci of the number of alleles ($N_a$), the allelic richness ($A_R$) standardized for sample size, and the expected and observed heterozygosity ($H_{exp}$ and $H_{obs}$) with standard errors of means (SEM) per population and per catchment for both taxa using the R packages adegenet v. 2.1.5 (Jombart, 2008; Jombart and Ahmed, 2011) and PopGenReport (function allel.rich). Furthermore, haplotype diversity $h$ was calculated as $h = n/(n-1)(1 - \sum x_i^2)$, with sample size $n$ and frequency of haplotype $x_i$ (Nei and Tajima, 1981) per population to describe mitochondrial genetic diversity. Inbreeding was estimated by using the fixation index $F_{IS} = (H_{exp} - H_{obs})/H_{exp}$. 

Fig. 1. Location of the study waters in the Palatinate Forest-North Vosges Biosphere Reserve at the French-German border (a). Green areas indicate the different zones of the reserve. Circles mark the studied streams and their colors show the taxa found. Population codes are given in Table 1. The shortest distances between all study sites are highlighted in the river network by thicker, dark blue lines. Catchments are given by the respective stream names (cp. Tab. 1). River Rhine is indicated by a thicker line. The dashed line marks the border between France and Germany. For better visibility and scale, the area with sites Stb, FiM and Mob in the Moder catchment is enlarged in b). The location of the biosphere reserve in the border area between north-west France and south-west Germany in central Europe is shown in c).
2.5 Genetic diversity standardization between taxa

To make the genetic diversity information from both species in a habitat-centered approach more accessible to regional managers who are rather inexperienced in using genetic data, a standardization was performed for the two diversity parameters \( H_{\text{exp}} \) and \( A_R \). The basic ideas here were that (1) in those streams where the genetic diversity values for both species are highest, the management priority should be strongly oriented towards conserving the current status, while for those waters where diversity values for both species are lowest, the focus should be on improvement measures and that (2) the minima and maxima of observed values \( \pm \) standard error of means (SEM)) reflect the actual framework of action of regional managers. The standardization was thus calculated as “scaled expected heterozygosity” \( s_{H_{\text{exp}}} = \frac{(H_{\text{exp}} - \min(H_{\text{exp}} - \text{SEM}))}{(\max(H_{\text{exp}} + \text{SEM}) - \min(H_{\text{exp}} - \text{SEM}))}*10 \) and likewise as “scaled allelic richness” \( s_{A_R} \), so that per species the highest unscaled value corresponds to 10 and the lowest unscaled value to 0. Bringing these values for both species on a uniform scale then allows a graphical representation that (1) focuses on the specific habitats, (2) presents both taxa equally weighted, and (3) equates the two reference frames for management. The essential purpose of the suggested standardization is to align the actual regional framework of management action between taxa. Additionally, correlations of the scaled values between the species were tested using Pearson’s product moment correlation coefficient (PPMCC; R function cor.test), including only sites without both taxa present, to elucidate whether the studied habitats as such have an influence on the genetic diversity.

### Table 1. Population codes, sampling locations, and number of analyzed specimens per marker type (NCR: control region sequences, NMS: microsatellites) and taxon. Catchments are ordered from south to north, which corresponds to the downstream direction of the receiving river Rhine. Populations within catchments are ordered in downstream direction, which also approximately reflects proximity.

<table>
<thead>
<tr>
<th>Catchment Population</th>
<th>Body of water</th>
<th>Coordinates (UTM 32M)</th>
<th>NCR Cottus spp.</th>
<th>NMS Cottus spp.</th>
<th>NCR Salmo trutta</th>
<th>NMS Salmo trutta</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zorn</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bab</td>
<td>Baerenbach</td>
<td>375447.15680; 5396564.6897</td>
<td>12</td>
<td>12</td>
<td>102</td>
<td>107</td>
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<tr>
<td>Fab</td>
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<td>377627.40796; 5405730.6837</td>
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<td>Fischbaechel (Zorn)</td>
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<td>12</td>
<td>12</td>
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<td>39</td>
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<tr>
<td><strong>Moder</strong></td>
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<tr>
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<tr>
<td>Ast</td>
<td>Aspenthal</td>
<td>394295.21662; 5421075.7575</td>
<td>36</td>
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<td><strong>Sauer</strong></td>
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<tr>
<td>Lgb</td>
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<tr>
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<td>Schmelzbach</td>
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<td>12</td>
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<td>35</td>
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<td><strong>Lauter</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Queich</td>
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<td>12</td>
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<td><strong>Deb</strong></td>
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<td>12</td>
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<td><strong>∑</strong></td>
<td></td>
<td></td>
<td>99</td>
<td>100</td>
<td>255</td>
<td>466</td>
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</table>

2.6 Genetic differentiation and structure

Differentiation of populations was measured by the number and frequency of private microsatellite alleles and their occurrence at the levels of loci, individuals and realized alleles using the private alleles function of the poppr v. 2.9.3 (Kamvar et al., 2014, 2015) R package. Also, the number of private alleles was standardized for sampling size by using a resampling approach: we used the smallest sampling size (sculpin: 12, trout: 15) to draw random individuals from each population and then determined the number of private alleles per population 100,000 times. The means of these reiterations per population provide an estimate of the number of private alleles that would have been expected if only the smallest sample size had been used in all populations, and thus allow direct comparisons between populations (cp. Kalinowski, 2004). Furthermore, we calculated pairwise Jost’s D (Jost, 2008) between all populations with the pairwise_D function of the mmd v. 1.1.3 (Winter, 2012) R package from regional managers. The standardization was thus calculated as”scaled expected heterozygosity” \( s_{H_{\text{exp}}} = \frac{(H_{\text{exp}} - \min(H_{\text{exp}} - \text{SEM}))}{(\max(H_{\text{exp}} + \text{SEM}) - \min(H_{\text{exp}} - \text{SEM}))}*10 \) and likewise as “scaled allelic richness” \( s_{A_R} \), so that per species the highest unscaled value corresponds to 10 and the lowest unscaled value to 0. Bringing these values for both species on a uniform scale then allows a graphical representation that (1) focuses on the specific habitats, (2) presents both taxa equally weighted, and (3) equates the two reference frames for management. The essential purpose of the suggested standardization is to align the actual regional framework of management action between taxa. Additionally, correlations of the scaled values between the species were tested using Pearson’s product moment correlation coefficient (PPMCC; R function cor.test), including only sites without both taxa present, to elucidate whether the studied habitats as such have an influence on the genetic diversity.
Table 2. Genetic diversity values of sculpin (*Cottus* spp.) and brown trout (*S. trutta*) from nine and eleven microsatellite loci respectively, per population and catchment (italic). Shown are mean number of alleles (*N_A*), mean allelic richness (*A_R*), mean observed (*H_{obs}*) and expected (*H_{exp}*) heterozygosity, and mean fixation index *F_{IS}*. Numbers in brackets are standard errors of means.

<table>
<thead>
<tr>
<th>Catchment population</th>
<th><em>C. spp.</em></th>
<th><em>S. trutta</em></th>
<th><em>C. spp.</em></th>
<th><em>S. trutta</em></th>
<th><em>C. spp.</em></th>
<th><em>S. trutta</em></th>
<th><em>C. spp.</em></th>
<th><em>S. trutta</em></th>
<th><em>C. spp.</em></th>
<th><em>S. trutta</em></th>
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<tbody>
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<td>Zorn</td>
<td>6.11 (1.09)</td>
<td>10.82 (2.42)</td>
<td>4.61 (0.70)</td>
<td>8.81 (1.89)</td>
<td>0.61 (0.09)</td>
<td>0.68 (0.06)</td>
<td>0.44 (0.08)</td>
<td>0.61 (0.06)</td>
<td>-0.02 (0.04)</td>
<td>0.10 (0.04)</td>
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<tr>
<td>Bab</td>
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<td>3.04 (0.51)</td>
<td>5.86 (1.01)</td>
<td>0.46 (0.07)</td>
<td>0.65 (0.05)</td>
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<td>Fab</td>
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<td>8.00 (1.69)</td>
<td>2.67 (0.33)</td>
<td>6.29 (1.19)</td>
<td>0.44 (0.07)</td>
<td>0.65 (0.06)</td>
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<td>0.43 (0.08)</td>
<td>0.66 (0.05)</td>
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<td>0.60 (0.07)</td>
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<tr>
<td>Moder</td>
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<td>2.86 (0.20)</td>
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<td>0.54 (0.04)</td>
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<td>Mob</td>
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<td>7.11 (1.02)</td>
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<td>5.46 (0.87)</td>
<td>5.46 (0.87)</td>
<td>0.66 (0.05)</td>
<td>0.66 (0.05)</td>
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<td>Sauer</td>
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<td>7.73 (1.80)</td>
<td>2.28 (0.35)</td>
<td>6.81 (1.17)</td>
<td>0.39 (0.08)</td>
<td>0.70 (0.05)</td>
<td>0.34 (0.07)</td>
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<td>0.74 (0.04)</td>
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<td>-0.02 (0.05)</td>
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<td>8.78 (1.86)</td>
<td>0.47 (0.07)</td>
<td>0.74 (0.04)</td>
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<td>0.71 (0.05)</td>
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<td>Sub</td>
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<td>2.40 (0.33)</td>
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<td>0.30 (0.08)</td>
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<td>0.13 (0.14)</td>
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</tr>
<tr>
<td>Deb</td>
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</table>
genetic clusters based on the Bayesian Information Criterion (BIC), which we then applied in a Discriminant Analyses of Principle Components (DAPC; Jombart, 2008; Jombart and Ahmed, 2011) to visualize population structure and differentiation. Finally, we constructed haplotype networks with the haploNet function (package pegas) to visualize mitochondrial population structure.

2.7 Genetic species identification

To allow species identification of sculpin based on mitochondrial haplotypes we further added the following reference sequences to the haplotype network:
- All 59 haplotypes from Volckaert et al. (2002), which provide the relevant haplogroups, later designated to species.
- Haplotype Cot65 from Šlechtová et al. (2004), as we retrieved sequences identical to their haplotype Cot66, which was found in the same location.
- Sequence AB188166 from Yokoyama and Goto (2005), found in C. poecilopus, as an outgroup.

3 Results

Sculpin were only present at nine out of 15 sites, while brown trout were found at all sites. Thus, the distribution of sculpin appeared fragmented in the region. Overall, the population genetic results also indicate a much stronger structuring of sculpin compared to brown trout. Genetic diversity in general was higher in brown trout than in sculpin.

The occurrence of null alleles was indicated for all nine microsatellite loci in sculpin, i.e., zero was not included within the 95% confidence interval of the bootstrap estimate, but when estimated per population null alleles were indicated for only two loci in four, and in one out of nine populations. In brown trout null alleles were indicated at ten out of eleven loci and the frequency was generally lower than in sculpin. The estimates per population showed only a few indications for the presence of null alleles at three loci, which appeared to be randomly distributed across populations (Tab. S2-1). Deviations from HWE were found in sculpin populations at all loci except one. Significant deviations ($p < 0.05$) for six loci were found in only one population each. For the other two loci deviations were found in two or four populations. The highest number of loci with deviations from HWE per population was three (Stb). In brown trout two loci showed significant deviations from HWE in more than half of the populations (eleven and eight, respectively). The number of populations with deviations ranged between one and five for the other loci. No deviation from HWE was found in one population (Flt). In the other populations the number of loci with significant deviations ranged between one (Brt, Sub) and six (Deb) loci (Tab. S2-2). Overall, these two indicators of the microsatellite data quality appeared to be randomly distributed across loci and populations, and therefore we believe they did not substantially affect our results.

3.1 Genetic diversity

In general, genetic diversity of sculpin was lower, compared to brown trout. The lowest values of mean allelic richness, and observed and expected heterozygosity found in brown trout populations were higher than the highest respective values found in sculpin populations (Tab. 2). The $A_R$ per population was lowest in FiM and highest in Brt, and lowest in Lauter and highest in the Zorn catchment in sculpin. In brown trout populations, the lowest $A_R$ was in the Brt population and the Sauer catchment and highest in FiM population and Moder catchment. The lowest $H_{exp}$ was in sculpin population FiM and brown trout population Flt, and the highest $H_{exp}$ was 0.46 in sculpin population Bab and in brown trout population Lib. Fixation index $F_{is}$ differed by > 0.1 from 0 in five of the nine sculpin populations, but only in one brown trout population, indicating possible inbreeding in several sculpin populations. Further details and additional genetic diversity values are given in Table 2.

Sequencing of the mitochondrial control region revealed seven haplotypes in sculpin and six haplotypes in brown trout. We found five previously undescribed haplotypes in sculpin and two in brown trout (Tab. S3-1). Haplotype diversity $h$ was considerably higher in brown trout [0.00 (Bab) to 0.78 (Stb)] than in sculpin (Tab. S3-2). Only in two sculpin populations, FiZ and Que, was $h$ larger than zero (0.41 and 0.30). The only brown trout population in which $h = 0$ was Bab, and the only haplotype found here was by far the most frequent haplotype, HTSail01.

Scaled genetic diversity values $A_R$ and $H_{exp}$ ranged from 0.84 (FiM) to 7.66 (FiZ), and 1.45 (FiM) to 8.36 (Bab) respectively, in sculpin populations. In brown trout populations these ranges were from 0.72 (Brt) to 7.62 (FiM), and 2.24 (Flt) to 8.70 (Lib) respectively (Fig. 2). We found only a very weak (PMMC: −0.41), statistically non-significant ($p = 0.280$) correlation for $A_R$, but a closer (PMMC: −0.83) and significant ($p = 0.006$) correlation for $H_{exp}$ between sculpin and brown trout populations.

3.2 Genetic differentiation and structure

Private alleles were found more frequently in sculpin than in brown trout (Tab. 3). In sculpin populations the range was between zero (Fah) and eleven (Bab). Standardized for sampling size, the corresponding estimated numbers ranged between 0.32 and 9.80. In four populations (Stb, Sub, Sch, Bab) all individuals carried at least one private allele. Except population Fah (no private alleles) the lowest number (4) of individuals with private alleles was in population FiM. In brown trout no private alleles were present in five (Ast, Brt, Que, Sch, FiZ) out of 15 populations and ranged between one and five in the other populations. Standardized private alleles ranged between 0.03 and 3.46. The number of individuals carrying private alleles in these populations was between one (Lgb) and ten (Deb, Sub).

The pairwise Jost’s D distances between sculpin populations were generally larger than between brown trout populations with nuclear and mitochondrial markers (Fig. 3, Tab. S2-4). The largest distance based on microsatellite data between sculpin populations was 0.78 (FiM-Stb, FiM-Lib).
The smallest distance of 0.26 was between FiZ and Fab in the Zorn catchment. Overall, sculpin populations FiM and Stb consistently had the largest genetic distances from other populations. In brown trout populations Flt and Brt appeared more genetically distant from the other populations. The highest distance was 0.27 (Flt-Brt). This was the only distance between brown trout populations, that was higher than the lowest distance between sculpin populations. All pairwise distances were highly significant \( p < 0.001 \), except between brown trout populations FiZ and Fab in the Zorn catchment \( p = 0.015 \), which was also the smallest distance between brown trout populations. Based on mtDNA data the largest distances between populations were 0.74 in sculpin (Scb-Stb) and 0.12 in brown trout (Scb-Sub). Distances of 0.00 were found between two sculpin and between six brown trout populations. Distances between sculpin populations were mostly statistically significant \( p < 0.05 \), but between brown trout populations less than half of the distances were significant (Tab. S2-4).

The analysis of molecular variance revealed that in bullhead (C. gobio) the differentiation within individuals contributed the most (47%) to the total variation followed by
the between-populations-within-catchments variation (32%). Between-catchments variation also contributed notably (18%). The contribution of the within-individual variation was very dominant (88%) in brown trout, while the other hierarchical sources of variation played only a minor role (Tab. 4). For bullhead the AMOVA of mtDNA data showed that differentiation between catchments contributed by far the most to the genetic variation (90%), while differentiation between populations within catchments and within populations is negligible (9% and 1%; Tab. 4). In contrast, differentiation between catchments is absent (<0%) in brown trout and within population differentiation accounts for most of the total variation (77%), but was not statistically significant (p = 0.751; Tab. 4).

Cluster analysis identified nine genetic clusters in sculpin and ten clusters in brown trout (Fig. 4). The DAPC showed considerable differentiation between genetic clusters in sculpin, but the clusters of brown trout formed a barely differentiated cloud. Only brown trout cluster 7 appeared somewhat separated. Cluster 7 consisted almost exclusively of trout from population Flt. With this exception, there were no clear relationships between brown trout genetic clusters and populations or catchments (Fig. 4, Fig. S4-1). In contrast, genetic clusters of sculpins corresponded very well with single populations and also catchments. Especially clusters 1 and 6, but also 2 and 9, appeared very distinct (Fig. 4, Fig. S4-2). A remarkable exception were clusters 1 and 7. These two clusters divided the population Bab, and cluster 1 was the most separated one. It was noticeable that all sculpin in cluster 1 were homozygous at locus Cgo1016 with an irregular (i.e., out of the repeat motif) allele (“136”) and this allele caused the population to separate into two genetic clusters. Sequencing of the locus showed a deletion in a series of eleven (allele “136”) thymin nucleobases adjacent to the (GT) n repeat motif (Fig. S4-3, base position 77), and the allele was thus handled as a normal allele.

The distribution of mtDNA haplotypes over populations appeared more differentiated in sculpin compared to trout. Three sculpin haplotypes (HTCot01, HTCot04, HTCot06) were found in more than one population, while the other four haplotypes were exclusive to a single population each (Fig. 5). Brown trout haplotypes appeared in at least two (HTSall05, HTSall06), and in up to eight (HTSall01) out of nine populations (Fig. S3-1).

### 3.3 Species identification and distribution

The comparison with the GenBank database and the haplotype network identified haplotype HTCot07 as *C. rhenanus* (Fig. 5). The 732 base pairs sequence was identical with haplotype HV18 from Volckaert et al. (2002) and with sequence MF326941 from Fast et al. (2017). The other six haplotypes were assigned to *C. gobio*. Haplotype HTCot07 was only found in population Sib in the Moder catchment and all twelve specimens examined in this population carried haplotype HTCot07 (ep. Fig. 4). Flow line distance between Sib and FlM is only approximately 3.1 km (Fig. 1b), and in population FlM we found the most common *C. gobio* haplotype HTCot06 exclusively.
4 Discussion

Our study revealed significant differences for conservation and management between sculpin and brown trout. First, brown trout were found in all streams, but sculpin (*C. gobio* and *C. rhenanus*) in only nine out of 15 streams. Apparently, sculpin populations are fragmented, while brown trout populations could be more closely connected. Therefore, we assume that gene flow between sculpin populations is interrupted, while it might be maintained, even over larger distances, between brown trout populations. Furthermore, we assume that both taxa must have been present in all suitable streams during the original colonization of the area. The present fragmented distribution of the sculpin probably results from local extinction events due to environmental stochasticity or anthropogenic damage events. The presence of brown trout in all streams may be explained by their better dispersal ability (including successful passage of minor barriers) and/or because they were restocked in affected areas (cf. below). Accordingly, special attention should be paid to the connectivity between sculpin populations in future stream management. However, it is essential to also consider the genetic integrity of the populations, or species.

4.1 Genetic diversity and inbreeding

Overall, microsatellite and mtDNA data showed that the genetic diversity of brown trout was higher compared to sculpin. Lowered genetic diversities may indicate small population sizes and/or inbreeding. Thus, the relatively low genetic diversity of sculpin populations was in accordance with the observed fragmented distribution (cf. Knaepkens et al., 2004;...
It is likely that most of the sculpin populations are isolated and small. Stronger deviations of \( F_{IS} \) from zero may be interpreted as indicative for inbreeding (\( F_{IS} > 0.1 \)) or outbreeding (\( F_{IS} < -0.1 \)). Sculpin population \( S_b \), the only \( C. rhenanus \) population (see below), had a low genetic diversity and \( F_{IS} \) was 0.23, so inbreeding could be relevant in this population. Hybridization with \( C. perifretum \) could possibly provide an alternative explanation, but the known hybrid lineage seems to be restricted to larger rivers, while small headwaters harbor pure populations of the parental species (Nolte et al., 2005; Stemshorn et al., 2011). Furthermore, we could not confirm the presence of \( C. perifretum \) in the region (cp. below). Other populations with \( F_{IS} > 0.1 \) include \( B_b \), which had the highest genetic diversity amongst sculpin and \( F_a \), where \( F_{IS} \) was negative (−0.13). Overall, we think inbreeding is currently not a major concern for sculpin populations in the biosphere reserve. However, increasing the presumably small population sizes may be helpful and an appropriate measure to prevent inbreeding in the long term. Depending on the specific situation, this could be achieved, for example, by limiting input of sandy sediments and facilitating in-stream erosion to prevent or remove clogging and thus provide more suitable nesting sites with unclogged stones and gravel. Another management option is the removal of barriers to adjacent suitable and unoccupied habitat (cf. Knaepkens et al., 2004). Both measures potentially increase the effective population size and thus counteract possible inbreeding. In brown trout population \( F_{IS} \) generally deviated lesser from zero, with the exception of \( B_r \) where \( F_{IS} \) was −0.16. We believe that in- or outbreeding is not currently of major concern for brown trout populations in the biosphere reserve either.

Our analyses of the mitochondrial control region discovered five previously unknown sculpin haplotypes and two new brown trout haplotypes. Since our study was the first study using this method in the region, this is not surprising, although a relatively large amount of comparable data is already available for both taxa in Europe (e.g., Volkova et al., 2002; Šlechtová et al., 2004; Stemshorn et al., 2011 (sculpin), and Cortey et al., 2009; Kohout et al., 2012; Lerceteau-Kohler et al., 2013 (brown trout)). In any case, these results highlight the important role that biosphere reserves can play in protecting genetic diversity.

The only brown trout population with \( h = 0 \) was the former donor population \( B_a \). The only haplotype found here was the by far most frequent haplotype HTSal01 (Tab. S3-2, Fig. S3-1). Ultimately, it remained unclear whether the use of population \( B_a \) as a donor for a brood stock contributed to the further spread of this haplotype in the region. Even though, in contrast, genetic diversity values of \( B_a \) based on microsatellites were not noticeably low, the brood stock itself should be evaluated if it is to continue to be used to produce fish for stocking.

### 4.2 Scaled genetic diversity

The genetic diversity of populations of different species, derived from different sets of microsatellite loci, cannot be compared directly. Even within a single species it can be problematic to compare absolute diversity values between regions or even studies (e.g. Schmidt et al., 2017). Consequently, it is difficult to evaluate such values, as it is necessary for management decisions. However, to arrive at optimal management at the local/regional level, it seems appropriate and purposeful to use all available values from the region as an “absolute” evaluation standard. Thus, we attempted to overcome these difficulties by bringing diversity values for both species on a uniform scale. At the same time, this approach should make it easier to rank streams, with equal consideration given to the genetic situation of both taxa, along a continuous axis of management priorities from “improve” to “conserve.”

No stream harboring both taxa could be assigned unambiguously to the “improve” range or the “conserve” range of the management spectrum (Fig. 2). This was also
Fig. 4. Discriminant Analyses of Principal Components (x-axis: PC 1, y-axis PC 2) of sculpin (Cottus spp., a) and brown trout (Salmo trutta, b). Lines between individuals and “group centers” show the genetic clusters. Larger/outer circles indicate the catchment and smaller/inner dots the population where the individual fish were sampled. Individual membership probabilities per cluster are shown in Fig. S4-1 and S4-2. For sculpin populations Bab and Stb additional asterisks show the mtDNA control region haplotypes. Haplotype HTCot01 was designated to C. gobio and HTCot07 to C. rhenanus (cp. Tab. S3-1).

Fig. 5. Network of sculpin (Cottus spp.) haplotypes. Pie charts represent haplotypes and their size shows the haplotype frequency. The colors of chart slices indicate the populations (cp. Tab. 1). The number of mutational steps between haplotypes is shown by the number of tick marks on connecting branches. Colored dashed and dotted lines mark the haplogroups I, II, III, IV and VII after Volckaert et al. (2002). Haplotype HTCot01, or Cot66 (Štechtová et al., 2004), was not included in Volckaert et al. (2002), but was positioned in between group I haplotypes. Haplotype HTCot07 equals haplotype HV18 in haplogroup III. Colored background areas indicate species designations. Haplogroups I and II plus the haplotypes HTCot01 to HTCot06 from this study were considered as C. gobio. Haplogroup III, including haplotype HTCot07/HV18 was considered as C. rhenanus. Under C. perifretum we included haplogroups IV and VII.
reflected by the absent \((\omega_{AR})\) or weak \((\omega_{Hexp})\) correlation between the scaled diversity values of the two taxa. Most closely, Bab and FlZ in the Zorn catchment could be assigned towards the “conserve” priority, albeit genetic diversities of the brown trout populations in these streams are more in the middle range of the spectrum of this species. The relation of the two scaled metrics to each other and within streams and between taxa is necessarily somewhat different, which resulted in further ambiguities. For example, FlZ, \(\omega_{AR}\) and \(\omega_{Hexp}\) differ only slightly, for Fab both values are nearly equal for brown trout, but differ for sculpin, and for Scb both values differ for both taxa (Fig. 2). However, these results showed that improvement is an adequate management option in all streams, as in all cases at least one taxon would benefit. But it also became apparent in which streams special care is needed to not worsen the situation of populations already in a genetically good condition, most obvious in population FiM. Interestingly, the scaled diversity values were lowest for brown trout populations Flt and Brt, and somewhat less pronounced in Ast and Lgb, where no sculpin were found. We suggest that (habitat) improvement should be the management priority for these streams.

Taken together, mitochondrial genetic diversity \(h\) of both taxa was low (zero) in Bab and high in FlZ. Analogous to the microsatellite evaluations, these two populations may be used to guide the path along an axis of management priorities from “improve” to “conserve”.

### 4.3 Genetic differentiation and structure

Altogether, genetic differentiation was more pronounced between sculpin populations and their genetic population structure followed, in contrast to brown trout, and to some extent the geographical stream structure. The number of private alleles, i.e., alleles found exclusively in a single population, indicates differentiation between populations, but also points, in a broader sense, to the genetic uniqueness of populations. Sculpin populations Bab, FiZ, Stb, and Scb stand out by a particularly high number of private alleles \((N_{PA},\) \(sN_{PA},\) \(N_{PAind})\). In populations Bab, Sib, Scb, and Sub every sampled sculpin carried at least one private allele, and in population Ltb over 90% \((N_{PAind},\) Tab. 3). Therefore, at least for these populations, the value of genetically unique population characteristics should be carefully considered, e.g., when planning measures to improve habitat connectivity. The generally lower number of private alleles in brown trout populations may be partially explained by the larger number of populations studied. Higher numbers of private alleles were found in populations Sib, FlM, Ltb, Sub, and Deb. However, the differences compared to the further populations were clearly less pronounced than with sculpin. Since we assume that the brown trout populations are much better connected (migratory behavior, less restricted by small in-stream-obstacles, less patchy distribution) than the sculpin populations, genetic uniqueness is rather of secondary importance here.

Pairwise Jost’s D genetic distances were also generally higher between sculpin populations than between brown trout populations. Especially sculpin populations Sib and FiM (both Moder catchment), but also Ltb, differed strongly from all other populations. In brown trout populations Flt and Brt stood out a bit. Again, these results underscore that genetic uniqueness should be weighed when (re-) connecting sculpin populations. For instance, populations Stb and FiM should not be connected in order to increase their genetic diversity, although they are geographically close to each other if there is an impassable barrier between them. A better alternative would be to consider whether opportunities can be created for these populations to reach unoccupied habitats to increase effective population size. In the Mob, also nearby, no sculpin was found. In case there are other sculpin nearby, these should also be genetically investigated before measures are taken.

The AMOVA also showed differences between taxa relevant to management (Tab. 4). The within-individuals component (heterozygosity; microsatellites) alone accounted for nearly 90% of the genetic variance of brown trout. The contributions of the higher levels, i.e., between individuals, between populations, and between catchments, were accordingly low. The AMOVA based on mtDNA also showed the lowest level, i.e., the within populations component, contributed most (77%). To keep intraspecies genetic diversity high, one of the common recommendations is to mix populations across catchment boundaries (e.g. Laikre, 1999; Baer et al., 2007). We expect such (sub-) catchment-based management strategies to be less effective in the case of the biosphere reserves brown trout. Genetic diversity conservation should focus more on individual populations to maintain the high diversity within individuals. In bullhead mtDNA variation between catchments contributed by far the most (90%) to the total variation but based on microsatellites variation between populations (32%) contribute considerably more to the total variation than variation between catchments (18%). Therefore, a strictly catchment-based management is a requirement, but would likely be insufficient to conserve the total genetic variation and a population-based strategy seems more purposeful.

In brown trout the DAPC revealed a more pronounced differentiation of population Flt in the Moder catchment than shown by the previous analyses. All trout from this stream were members of the same genetic cluster (cluster 6) with a probability of 100%, and only very few trout from other streams were assigned to this cluster (Fig. S4-1). All other clusters were composed of fish from several, sometimes all (except Flt), streams. Thus, there was almost no apparent relation between genetic population structures and the stream system, and therefore catchment-based, geographic conservation units could hardly be established here, as it was possible for Mediterranean brown trout in major catchments in the Latium region in Central Italy (Rossi et al., 2022), for example.

Stocking can cause genetic homogenization of populations (e.g. Klütisch et al., 2019; Fitzpatrick et al., 2020), and thus can explain the low differentiation between the brown trout populations. Our sampling sites were selected to minimize potential effects of stocking, but stocking, also in connected streams, cannot be completely ruled out, as demonstrated by an unexpected observation of a single “tiger trout” (Salmo trutta x Salvelinus fontinalis; total length 132 mm) during our sampling in FlZ. On the other hand, stocking can also be without genetic consequences in a given stream, if stocked fish migrate out, are caught, or die before being reproductive (e.g. Ferguson, 2006).
In sculpin, the DAPC further highlighted the genetic population structures shown by the previous analyses. Populations Sib and Scb formed exclusive clusters and population FiM an almost exclusive cluster (Fig. S4-2). Interestingly, the two most widely separated clusters (clusters 1 and 7) could be attributed to different causes, which imply different consequences for the management. Cluster 1 was formed exclusively from sculpin with an irregular allele at one locus (all specmen homozogous). This allele could be related to a single deletion in the flanking region of the microsatellites’ repeat motive. Therefore, we believe that it shows a rather recent, unique mutation event and was inherited only partially within population Bab since then. Thus, the cluster probably indicates a family structure. Accordingly, the implications for management are not as serious as they might initially appear based on the very strong separation of cluster 1 alone. We think it is both necessary and sufficient to ensure the conservation status of the Bab population in the long term, in order to enable its possible independent evolutionary development. The cluster 7 was exclusive to population Stb. Analyses of mtDNA concluded that this population is a C. rhenanus population, whereas all other sculpin populations were bullhead, i.e., C. gobio. Accordingly, the population Sib is the only known occurrence of the species C. rhenanus in the study area to date (see below), and it is imperative that this species status be adequately addressed in future management of the biosphere reserve. It is remarkable that a single point mutation on a single microsatellite locus led to the formation of a cluster (cluster 1, C. gobio) that appeared more differentiated from conspecific clusters than a cluster of a second species (cluster 7, C. rhenanus). This demonstrates the sensitivity of the cluster analyses, but urges caution in deriving management implications, which differed for the two clusters in our case.

4.4 Sculpin taxonomy and distribution

The discovery of C. rhenanus in the headwater Stb in the Moder catchment was rather unexpected, because to the best of our knowledge this species was unknown in the Palatinate Forest-North Voges Biosphere Reserve before and does not occur in any of the five studied catchments according to IUCN distribution data (Freyhof and Kottelat, 2008a). The closest catchments with occurrences are the Speyerbach catchment, which directly borders the Queich catchment to the north, and the western drainages of the Northern Voges and Palatinate Forest (Freyhof and Kottelat, 2008a). In the study area, on the other hand, occurrences of C. perifretum could have been expected in addition to C. gobio based on IUCN distribution data (Freyhof and Kottelat, 2008b; Freyhof, 2011). Compared to the large number of small streams in the study area, our sample size of 15 stream sections was relatively small. Considering that the shortest distance between C. rhenanus and C. gobio populations was only about 3 km, it seems all the more likely that extended fine scale surveys, including the western drainages, would find further occurrences of C. rhenanus. Occurrences of C. perifretum in the region can furthermore not be ruled out. Our study focused on headwaters and this species may be found further downstream (cf. Nolte et al., 2005; Stemshorn et al., 2011). Cottus rhenanus has only been given species rank after the last amendment of EU Habitats Directive (Council Directive 92/43/EEC) Annex II. It was split from C. gobio, which is listed under the Annex II, and thus should inherit the same protective status.

Interestingly, our newly found C. gobio-haplotypes HTCot02, HTCot03, HTCot04, and HTCot05 grouped somewhat together, and apart from the two most common haplotype HTc01 and HTc006 in the network, and these haplotypes occurred exclusively in single streams or catchments (Fig. 5, Tab. S3-2). It is possible that these haplotypes are exclusive to the Palatinate Forest-North Voges region and are therefore advised to be specially protected. Probably, the taxonomy of European freshwater sculpin, especially the “C. gobio-complex” (or haplogroups I/II), is not conclusively resolved (Freyhof et al., 2005, cf. Stemshorn et al., 2011). Our findings suggest that further investigating the sculpin in the wider Upper Rhine region may be an important contribution to overcome taxonomic uncertainties and thereby may help to further improve the freshwater biodiversity management with regard to this fish genus.

4.5 Perspective

Our study provides fundamental information on intraspecific genetic variation of two characteristic freshwater fish in headwaters in a protected area. We found pronounced differences between the taxa relevant for management and conservation, and suggested exemplified approaches to adequately consider genetic diversity and structure in the conservation management of freshwater habitats at a regional scale. Our attempt to combine genetic information of taxa indicated possible ways to ensure and improve the protection of genetic diversity in a protected area in a habitat-oriented manner in the future. We derived stream-specific and species-specific recommendations for the Palatinate Forest-North Voges Biosphere Reserve, but the examples and approaches could be useful and further developed in other areas and/or with other taxa to facilitate management decisions.

We used standard metrics to describe the genetic diversity (richness and evenness), genetic differentiation (number of units and differentiation between units), and inbreeding (cp. Hoban et al., 2022). Therefore, our genetic composition data may not only be of use regionally, but may also be considered in larger contexts, as needed to improve biodiversity conservation policies. Furthermore, standardized monitoring of genetic diversity, in contrast to other biodiversity components, is currently carried out by very few countries, although such monitoring could contribute significantly to global biodiversity conservation (Hoban et al., 2022). This study provides a possible contribution to monitor genetic diversity in the biosphere reserve or in large-scale analyses.

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Supplementary Material

Supplemental Information S1. Locus and primer information (Tab. S1-1), PCR protocols (Tab. S1-2) and cycling parameters (Tab. S1-3) of six multiplex PCRs for microsatellite analyses. PCR protocols (Tab. S1-4) and cycling parameters (Tab. S1-5) for the sequencing of microsatellite locus Cgo1016. PCR protocols (Tab. S1-6) and cycling parameters (Tab. S1-7) for mtDNA control region amplification.

Supplemental Information S2. Null allele estimates (Tab. S2-1), Hardy-Weinberg equilibrium tests (Tab S2-2) and pairwise Jost's D distances based on microsatellite (Tab. S2-3) and mtDNA sequencing (Tab S2-4).

Supplemental Information S3. Cottus spp. and Salmo trutta haplotype (Tab. S3-1), haplotype diversity h (Tab. S3-2), and network of Salmo trutta haplotypes (Fig. S3-1).

Supplemental Information S4. Discriminant Analysis of Principal Components membership probabilities to genetic clusters (Fig. S4-1, Fig. S4-2) and alignment of sequenced microsatellite locus Cgo1016 (Fig. S4-3).

The Supplementary Material is available at https://www.kmae-journal.org/10.1051/kmae/2023005/olm.

References
