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ARTICLE

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Cryptic persistence and loss of local endemism in Lake Constance charr subject to anthropogenic disturbance

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Abstract

In the welcome circumstance that species believed extinct are rediscovered, it is often the case that biological knowledge acquired before the presumed extinction is limited. Efforts to address these knowledge gaps, in particular to assess the taxonomic integrity and conservation status of such species, can be hampered by a lack of genetic data and scarcity of samples in museum collections. Here, we present a proof-of-concept case study based on a multidisciplinary data evaluation approach to tackle such problems. The approach was developed after the rediscovery, 40 years after its presumed extinction, of the enigmatic Lake Constance deep-water charr *Salvelinus profundus*. Targeted surveys led to the capture of further species and additional sympatric normal charr, *Salvelinus* cf. *umbla*. Since the lake had been subject to massive stocking in the past, an evaluation of the genetic integrity of both extant forms was called for in order to assess possible introgression. A two-step genomic approach was developed based on restriction site associated DNA (RAD). Diagnostic population genomic (single nucleotide polymorphism [SNP]) data were harvested from contemporary samples and used for RNA bait design to perform target capture in DNA libraries of archival scale material, enabling a comparison between extant and historic samples. Furthermore, life history traits and morphological data for both extant forms were gathered and compared with historical data from the past 60–120 years. While extant deep-water charr matched historical deep-water specimens in body shape, gill raker count, and growth rates, significant differences were discovered between historical and extant normal charr. These resulted were supported by genomic analyses of contemporary samples, revealing the two extant forms to be highly divergent. The results of population assignment tests suggest that the endemic deep-water charr persisted in Lake Constance during the eutrophic phase, but not one of the historical genomic samples could be assigned to the extant normal charr taxon. Stocking with non-endemic

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charr seems to be the most likely reason for these changes. This proof-of-concept study presents a multidisciplinary data evaluation approach that simultaneously tests population genomic integrity and addresses some of the conservation issues arising from rediscovery of a species characterized by limited data availability.

KEYWORDS
Arctic charr, deep-water form, RAD-based genomics, *Salvelinus profundus*, stocking

INTRODUCTION

The correlation between species extinction and anthropogenic impacts is well documented, with thousands of species went extinct in the past and many more are estimated to disappear in the near future (Barnosky et al., 2011). Recently, Martin et al. (2022) identified more than 500 terrestrial vertebrate species as lost, because they have not been sighted for more than 50 years. While it is likely that most of these species are extinct, in the event that some are rediscovered there will be a need for urgent re-evaluation of their conservation status. Examples of recent rediscoveries include the Juan Fernández fur seal (Hubbs & Norris, 1971) and the mouse opossum *Cryptonanus ignitus* (Teta & Díaz-Nieto, 2019). Thorough documentation of such rediscoveries is crucial, especially in the light of current taxonomy but such studies are often hampered by lack of available information (Teta & Díaz-Nieto, 2019). The most important resources in integrative taxonomic approaches for documenting rediscovered species are usually scientific research collections. For many years, the most important data available from museum specimens concerned morphological characteristics, but recent advances in DNA sequencing technologies have enabled researchers to make use of scientific collections as archives of genetic information (Agne et al., 2022; Hahn et al., 2022; Rohland et al., 2004; Straube et al., 2021).

Here, we make use of both contemporary and archival DNA sequence information to document and critically analyze the recent rediscovery (Alexander et al., 2016) of the endemic deep-water charr of Lake Constance, *Salvelinus profundus* (Schillinger, 1901) hereafter referred to as the "deep-water form" or "deep-water charr" (Freyhof & Kottelat, 2005). Due to the presence of the morphologically similar Alpine charr, *Salvelinus* cf. *umbla* (Linnaeus, 1758) (hereafter referred to as "normal form" or "normal charr") in the lake, there is an urgent need for re-evaluation of the taxonomic and conservation status of the rediscovered deep-water form. The potential for gene flow between charr forms and species means this is a complicated task (Brachmann et al., 2021) made still more challenging by phenotypic plasticity and anthropogenic factors, including well-established but undocumented stocking practices (Englbrecht et al., 2002) and habitat alterations that may further obscure the original pattern of the autochthonous diversity in a given ecosystem.

The existence of two Arctic charr forms in Lake Constance was documented scientifically more than 100 years ago (Dörfel, 1974; Schillinger, 1901). The normal pelagic form reaches a total length (TL) between 20 and 45 cm and spawns during winter at depths between 50 and 100 m. In contrast, the benthic-feeding deep-water dwarf form attains a maximum TL of only 27 cm and spawns during summer at depths between 70 and 150 m. Populations of both forms declined dramatically in the 1970s and 1980s as a consequence of anthropogenic eutrophication of Lake Constance (Eckmann & Rösch, 1998). Restrictive water protection legislations implemented in the 1960s and the installation of numerous sewage treatment plants led to the restoration of oligotrophic conditions more than 40 years ago (Baer et al., 2017), but it appeared that these restorative measures came too late for the deep water dwarf form, which was reported extinct in 2005 (Freyhof & Kottelat, 2005). It was assumed that only the normal form persisted (Freyhof, 2009), in alpine and pre-alpine lakes of France, Switzerland, Germany, and Austria where it is classified as 'least concern' (Freyhof & Kottelat, 2008). It was therefore a great surprise when in 2011 a single charr phenotypically similar to historical specimens of the deep-water form was discovered in a bottom gillnet set by a commercial fisherman in the western part of Lake Constance (J. Behrmann-Godel and A. Sulger, University of Konstanz, personal communication). An extensive scientific fishing campaign in 2014 yielded further seven specimens (Alexander et al., 2016). These eight fish were the first deep-water charr recorded in Lake Constance since their presumed extinction more than 40 years ago.

The discovery prompted a discussion about the taxonomic identity and conservation status of the deep-water charr. Lake Constance had been heavily stocked for decades with charr from different provenances in order to compensate for reduced reproduction caused by

eutrophication of the lake. Furthermore, heavy phosphorus concentrations from intensified agriculture and human population growth in the 1960s and 1970s led to algal blooms and consequent oxygen depletion in the deep-water benthic zone of the lake (Nümann, 1972). Arctic charr yields declined dramatically, with only 70 kg of charr caught annually by professional fisherman between 1976 and 1981, compared to an average of around 1000 kg per year before eutrophication (Hartmann, 1984). From the mid-1970s onward, to reverse the trend of declining charr stocks, fishery managers stocked the lake with hundreds of thousands of juvenile charr of various foreign provenances (Hartmann, 1984), but unlike other systems where the knock-on effects of stocking were monitored (Lamaze et al., 2012; Marie et al., 2010), the impact on autochthonous charr diversity in Lake Constance was never evaluated. The threat of allochthonous introgressive hybridization was real (Savary et al., 2017), as was the possibility of complete replacement of the original form by stocked varieties (Englbrecht et al., 2002), but the genetic or phenotypic effects of stocking on extant charr populations in Lake Constance remained unknown. This lack of evaluation is a serious hindrance for conservation-oriented fishery management because fishing bans or proposals for large protection areas are generally considered justifiable only if endemic forms persist in an uncompromised state and are not replaced by stocked allochthonous individuals. Following the accidental rediscovery of the deep-water charr in Lake Constance, information regarding the taxonomic status and biology of both forms became essential in unpicking the ancestry and autecology of extant phenotypes and informing conservation decisions made by policy makers and stakeholders.

Preliminary analysis of the genetic distinctiveness of the first eight rediscovered deep-water charr specimens (Alexander et al., 2016) and comparison with seven extant normal charr specimens suggested that the deep-water charr has remained genetically differentiated from the normal charr in Lake Constance (Doenz & Seehausen, 2020).

In this study, we aimed to achieve a representative, population level sample size of the rare extant deep-water charr, based on targeted fishing in grounds historically recorded as productive for the form or as spawning grounds. Morphological features, life history (spawning time, feeding strategy, etc.) data were collected for both forms and compared with data derived from historical museum collection specimens. In addition, charr monitoring data from between 1980 and 1998 were interrogated in detail.

In order to achieve population genetic level evaluation of the autochthonous genetic integrity status of the rediscovered deep-water and normal charr, we tested extant specimens of both forms for diagnostic distinctiveness using restriction site associated DNA (RAD) sequencing. The resulting dataset was then used to design diagnostic RNA baits for target capture of informative loci in DNA from archival scale samples of deep-water charr (80–150 years of age) and to analyze the degree of divergence between archival and extant material. This methodological approach was selected in anticipation that it may serve as a blueprint for conservation genomic assessments in future studies where archival samples are used to critically test levels of extant autochthonous genetic integrity in rediscovered, rare, and cryptic species. Specifically, we aimed to (a) evaluate the probability that both endemic deep-water and normal forms of charr persisted during the eutrophic phase of Lake Constance; and to (b) assess the genotypic and phenotypic consequences of historical stocking for both forms.

MATERIAL AND METHODS

Contemporary fishing campaign in the study area

Lake Constance has a total surface area of 536 km^2 and is divided into a large (472 km^2), deep ($z_{max} = 254$ m, $z_{mean} = 101$ m), warm-monomictic Upper Lake (ULC) and a small (63 km^2), shallow ($z_{mean} = 16$ m) Lower Lake (Figure 1). The current distribution of charr occurs mostly in the deeper ULC (Alexander et al., 2016), which lies between Austria, Germany, and Switzerland (9°18′ E, 47°39′ N; Figure 1), while in the lower lake captures of charr are a rare exception. A minimum of 30 fish species live in the lake (Eckmann & Rösch, 1998), of which about 10 are targeted by professional fisheries (Rösch, 2014). During the times of most recent sampling around 100 commercial fishermen were operating on Lake Constance, with whitefish (*Coregonus* spp.) being the economically most important species. In ULC, fisheries management is based on routine monitoring of whitefish and other species (more details in Baer et al., 2017). Between November 2014 and November 2017 a total of 12 fishing campaigns were conducted in ULC, four during summer (July to mid-September) and eight in autumn (October to early December). Benthic gill nets with mesh sizes of 15, 17, 19, 20, 22, and 26 mm (each 33–120 m long and 2.5–7 m high) were set at three different locations at depths between 75 and 100 m. Location 1 (Figure 1), "Teufelstisch" (47°75′39.35″ N, 9°12′54.40″ E), is a well-known spawning ground for the normal charr form; location 2 (Figure 1), "Fließhorn"

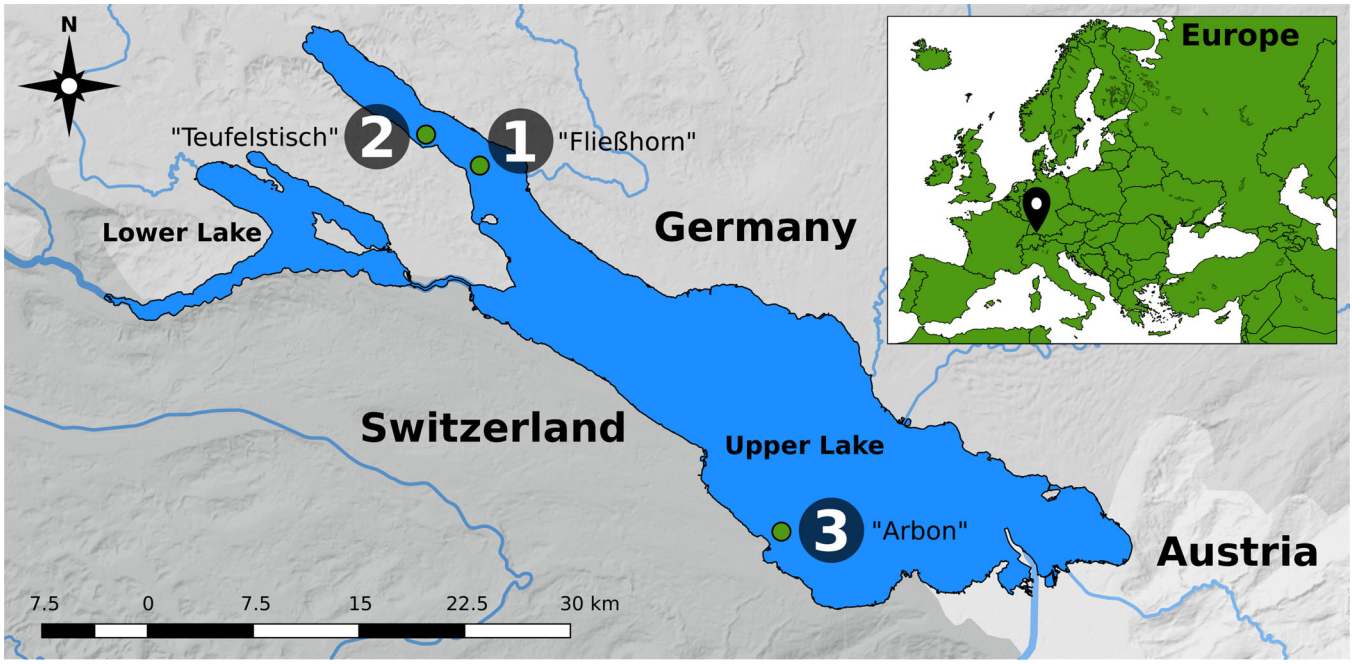

FIGURE 1 Map of Lake Constance with circulated numbers showing sampling locations.

(47°73′44.35″ N, 9°17′90.66″ E), was formerly regarded as a typical fishing ground for both forms of charr (Dörfel, 1974); and location 3 (Figure 1), "Arbon" (47°51′14.22″ N, 9°47′28.40″ E), was once a successful location for fishing the deep-water form (Hartmann, 1984).

Initial measurements from contemporary sampling

All charr caught in the gill nets were euthanized by an overdose of clove oil (*Caryophylli floris aetheroleum*, 1000 ppm) according to the German Animal Protection Law (§ 4) and the ordinance on slaughter and killing of animals (Tierschutzschlachtverordnung § 13). Thereafter, specimens were transported to the Fisheries Research Station at Langenargen and identified as either deep water or pelagic form according to the protocol of Dörfel (1974). A digital photograph (Pentax K-1II, 36.46 megapixels) was taken on the left side of each fresh fish and the TL and body mass (BM) were measured to the nearest mm and g, respectively. In addition, eye diameter (ED, measured as a horizontal line from the anterior to the posterior edge of the eye) and head length (HL, from snout tip to posterior point of the operculum) were recorded with a caliper to the nearest 0.1 mm. Gonado-Somatic-Index (GSI) was calculated for each mature charr as wet gonad weight as a percentage of wet body weight (total weight). Gill rakers were counted on the first left gill arch of every captured specimen. Scales were taken from the area between the adipose fin and the anal fin, outside the lateral line (Frost, 1978) in order to estimate age, and the sagitta otoliths were dissected from the first 10 captured individuals of each morph. Gastrointestinal tracts (stomach and intestine) were taken from 37 deep-water and 45 normal charr. Food items were identified and counted in a zooplankton counting chamber and categorized into five groups for comparison with historical samplings (Dörfel, 1974) as follows: pelagic zooplankton (*Bosmina, Copepoda, Daphnia/Diaphanosoma, Bythotrephes longimanus* and *Leptodora kindtii*), benthic macroinvertebrates (*Gammaridae, Dreissena* spp.), *Turbellaria* (cocoons), *Chironomidae*, and fish (*Perca fluviatilis*, *Cyprinidae*). The Cochran–Mantel–Haenszel test (Zhang & Boos, 1997) was used to screen for differences in dietary composition between historical (Dörfel, 1974) and contemporary specimens. To estimate the von Bertalanffy growth parameters of contemporary specimens, the non-linear least squares method was used to fit a growth curve (King, 2013). Fin-clip samples were taken from the caudal fin and stored in ethanol (Chemsolute, 99.5%, Th. Geyer, Germany) for further genetic analysis.

Data from 1980–1998

Between 1980 and 1998, a total of 1822 charr were sampled from Lake Constance at the behest of J. Hartmann (deceased 1998), a researcher at the Institute for Lake

Research of Baden-Württemberg, located on the German shore. TL, sampling location, and sampling depth was recorded for all specimens; and total wet weight, gill raker number, position of lower jaw (subinferior or subterminal), maturity, sex, and main stomach content (categorized as above) were recorded for the majority of specimens. Age was recorded for a minority ($n = 140$). An additional differentiation was made between normal and deep-water charr over the first 3 years (1980–1983) of this historical study, but this was discontinued thereafter. Hartmann also kept records regarding the number of gill rakers in stocked fish of foreign origin. In order to make compassion with contemporary data, this hitherto entirely unevaluated and unpublished data-set was transferred into an electronic database. For downstream analyses based on historical descriptions (Dörfel, 1974) and these authors' personal observations (i.e., time of maturity, TL, position of lower jaw), each individual was assigned to one of the two forms. Individuals with TL less than 300 mm, an upper jaw that overlapped the lower jaw (subinferior mouth) and gill rakers numbering between 19 and 27 and/or exhibiting maturity during summer were classified as a deep-water charr. Those with a lower jaw not overlapped by the upper jaw (subterminal mouth), immature during summer or with a TL greater than 300 mm were classified as normal charr. All other individuals were classified as "undetermined." Next, the food composition for normal and deep-water charr were determined and possible differences between historical (Dörfel, 1974) and contemporary samplings were analyzed using the Cochran–Mantel–Haenszel test (Zhang & Boos, 1997).

Influence on gill raker number

According to the records of Hartmann, from 1977 until at least 1992, thousands of normal charr of varying provenance (e.g., Sweden, Switzerland, Bavaria) and exhibiting significantly lower gill raker numbers than endemic normal charr (Dörfel, 1974) were stocked into Lake Constance. After 1992, most stocking involved offspring of charr caught in the lake. To test if those stockings had an influence on the gill raker number of normal charr in the lake, the intensity of stocking with *Salvelinus* cf. *umbla* between 1976 and today was recorded from annual reports of the IBKF (Internationale Bevollmächtigtenkonferenz für die Bodenseefischerei, or International Conference of Plenipotentiaries for Fisheries in Lake Constance, see Baer et al., 2017). The development of gill raker number between 1980 and 1998 was analyzed with the following general linear model (GLM) (Sachs, 1997):

$$Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + (\alpha\beta)_{ij} + (\beta\delta)_{kl} + \varepsilon_{ijklm} \quad (1)$$

where Y_{ijklm} is gill raker number in the normal form; μ is the overall mean, α_i denotes the annual yield of charr in Lake Constance (obtained from the IBKF), β_j is annual stocking intensity (of yearlings), γ_k is the mean oxygen level above the lake bottom during the period of egg incubation (January to March, in mg/L), δ_l refers to concentrations of phosphorous (P) measured during winter mixing (February–March), $(\alpha\beta)_{ij}$ is the interaction between yield and stocking density, $(\beta\delta)_{kl}$ is the interaction between P and stocking density, and ε_{ijklm} is the random residual error. Data for P and oxygen were kindly provided by the Institute of Lake Research, LUBW, Germany.

Geometric morphometrics from contemporary and conserved samples

In order to compare the ED between normal and the deep-water charr, HL from contemporary samples was corrected for size according to the following formula (Elliott et al., 1995):

$$M_s = M_0 \cdot (L_s/L_0)^b \quad (2)$$

where M_s = size-corrected parameter, M_0 = measured parameter length, L_s = mean TL of all examined individuals, L_0 = TL of the individual, b = slope of the regression of log M_0 on Log L_0. HL was incorporated into the following GLM (Sachs, 1997):

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + \delta_l + \varepsilon_{ijkl} \quad (3)$$

where Y_{ijkl} is ED; μ is the overall mean, α_i denotes HL corrected for size, β_j is TL, γ_k, is sex (male, female, juvenile), $(\alpha\beta)_{ij}$ is the interaction between HL corrected for size and TL, δ_l is form (deep-water or normal), and ε_{ijkl} is the random residual error. The Tukey–Kramer HSD (honestly significant difference) test was used for post hoc comparisons between sexes (Hayter, 1984).

The influence on HL was tested with the following GLM:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + \varepsilon_{ijk} \quad (4)$$

where Y_{ijk} is the HL; μ is the overall mean, α_i denotes form (deep-water or normal, HL corrected for size), β_j is sex (male, female, juvenile), γ_k is body length (in mm), $(\alpha\beta)_{ij}$ is the interaction between TL and sex, and ε_{ij} is the random residual error. The Tukey–Kramer HSD test was used for post hoc comparisons between sexes and forms

(Hayter, 1984). All statistics were run on JMP Pro 15.2.1 (64 bit, SAS Institute).

In order to perform geometric morphometrics (GMM) using landmarks, digital images from all contemporary samples selected for genetic analysis were first checked for accuracy. Images of fish with inflated abdomens, broken maxillae, or other damage (due to handling during the fishing procedure) were excluded from the data set. Only specimens from locations where both forms occurred sympatrically were chosen. To account for allometric effects on body shape, only specimens of closely comparable length (200–250 mm) were selected. As a result of this selection, 36 images of the deep-water form and 28 images of the normal form were used for the final landmark analysis. In addition, digital images of charr preserved in different museums (examples see Appendix S1: Figure S1) were added to the GMM, including normal form specimens preserved in 1882 by C.B. Klunzinger and stored in the Stuttgart State Museum of Natural History, Germany (lot SMNS 25550) and deep-water individuals preserved in 1962 by N. Peters and stored in the California Academy of Science, San Francisco, USA (lot CAS-ICH-209135). The digital images were checked for obvious deterioration due to preservation prior to analysis, resulting in four normal charr and four deep-water charr being used in the final analysis. Two individuals of the deep-water form preserved in 1901 by A. Schillinger and stored in the Bavarian State Collection of Zoology, Munich, Germany (lot ZSM-PIS-30623), were rejected due to apparent deformation and shrinkage.

The tpsDig2 software (V 2.32, F. James Rohlf, James Rohlf, Stony Brook University, New York, USA) was used to digitize 16 landmarks (Figure 4a) on each chosen digital image. In order to identify differences in body shape between the two forms, the landmarks were evaluated using the program PAST 4.0 (Hammer et al., 2001). In a first step, the landmarks were made comparable with the help of a "Procrustes-Superimposition" (Stegmann & Gomez, 2002). The obtained Procrustes coordinates were then used to perform a principal component analysis (PCA; Sokal & Rohlf, 2003) and the first two principal components (PC) were selected to generate a scatter plot. A linear discriminant analysis (LDA; Sokal & Rohlf, 2003) was carried out to determine which variables best distinguished the two forms. The first two linear discriminants (LD) were selected to generate a scatter plot and appropriate allocation of individuals to the respective forms was checked using a Jackknife cross-validation test (Fan & Wang, 1996). The LDA was then repeated without grouping the historical samples and a Jackknife cross-validation was repeated to assign the historical samples to one of the two forms. Finally, a pairwise discriminant function analysis (DFA) was performed, coupled with a permutation test (10.000 runs) in MorphoJ [V1.07a; (Klingenberg, 2011)] in order to compare the Procrustes distances of contemporary and historical individuals from both charr forms.

DNA extraction

Genomic DNA was extracted from fin clips of 96 individuals from extant *Salvelinus* caught in Lake Constance between 2014 and 2017 (48 samples from each charr form) by Eurofins Genomics (Ebersberg, Germany) using the Qiagen DNeasy Blood & Tissue kit. At least 1 mg of DNA was sent for RAD Library preparation and sequencing following the protocol of Baird et al. (2008) to Floragenex, Portland, Oregon, USA.

Historical DNA for genetic analyses was sourced from 14 archived scale samples from Arctic charr collected at irregular intervals by the Institute for Lake Research of Baden–Württemberg, LUBW, Germany between 1931 and 1937 ($n = 11$) and between 1954 and 1957 ($n = 3$). This material had been air-dried and stored separately in paper envelopes. Since most of the envelopes were labeled simply "charr" they were tentatively assigned a priori to either normal ($n = 5$) or deep-water ($n = 9$) forms based on metadata written on the envelopes (i.e., date, depth of catch, TL, sex, and maturity status) using the same criteria applied for species differentiation in the records of Hartmann. DNA was extracted from the scale samples ($n = 14$) by Microsynth AG, Balgach, Switzerland. Two samples (both normal charr) were later excluded from the data set due to low DNA-quality, but the remaining 12 extracts were sent to Arbor Biosciences for bait RAD locus enrichment via hybridization-based capture (Ann Arbor, Michigan, USA).

RAD sequencing data and population genomic analysis of extant samples

SbfI-RAD-Seq library preparation was carried out on extracts of *Salvelinus* DNA by Floragenex (Portland, Oregon, USA). One 95-plex library containing all recently collected charr individuals from Lake Constance was sequenced, and the RAD loci were then filtered for quality. Loci with variant sites were filtered for population descriptive analysis and coancestry analysis. More information about these analytical steps is provided in Appendix S2.

RAD locus enrichment and population assignment of historical samples via hybridization-based capture

Based on the output of locus-specific F-statistics of the *refmap.pl* module of the *stacks 2.4*.-RAD-analysis pipeline (Catchen et al., 2013), all RAD locus sequences were ranked according to locus-specific Fst-Values between deep-water and normal charr. One hundred and eighty two loci featuring the highest Fst-Values and having only one single nucleotide polymorphism (SNP) were selected in the first instance, then those showing a high level of similarity (similarity threshold 86%) in nucleotide sequences were excluded. Sequence information for the remaining loci ($n = 164$) was retained and forwarded to the Arbor Biosciences Lab (Ann Arbor, Michigan, USA) along with DNA-extracts from historical scale samples for use in bait design and target capture. Sixty five loci were excluded prior to RNA bait production after failing quality measures for bait design. Ninety nine loci were finally chosen for bait production and baits were hybridized to these 99 targets in double stranded DNA libraries constructed from DNA extracts followed by amplification of captured libraries and sequencing. Sequence reads were subsequently mapped to the *Salvelinus alpinus* reference genome [GenBank assembly accession number: GCF_002910315.2; (Christensen et al., 2018)]. Finally, all retained reads from all 14 samples were combined for downstream analysis with previously retained RAD-reads of extant specimens and mapped to the 99 bait RAD locus sequences. These sequences were used to identify individual-based patterns of population differentiation and population assignment within extant charr from Lake Constance. To this end, a standardized distance-based principal coordinates analysis (PCoA) was performed in GeneAIX 6.503 (Peakall & Smouse, 2006, 2012) based on the codominant genetic distance matrix (Nei's standard genetic distance) generated from 89 SNPs and genotyped for 40 extant normal-type charr, 43 extant deep-water charr and 12 (out of 14) successfully genotyped historical *Salvelinus* samples. To infer the number of genetic clusters in the 89 diagnostic SNP data set and to assign individuals to a given number of populations, a model-based Bayesian approach allowing for admixture between populations was applied as implemented in *STRUCTURE* 2.3. (Pritchard et al., 2000) in combination with the ΔK method (Evanno et al., 2005). All analytical steps are listed in Appendix S3.

RESULTS

Contemporary sampling

The 12 fishing campaigns that took place between 2014 and 2017 yielded a total of 364 captured charr (Appendix S4: Table S1). According to Dörfel (1974), it is possible to unambiguously identify the deep-water charr form by morphological characteristics such as the subinferior mouth and the notably smaller lower jaw, which can be overlapped by in the upper jaw (Figure 2).

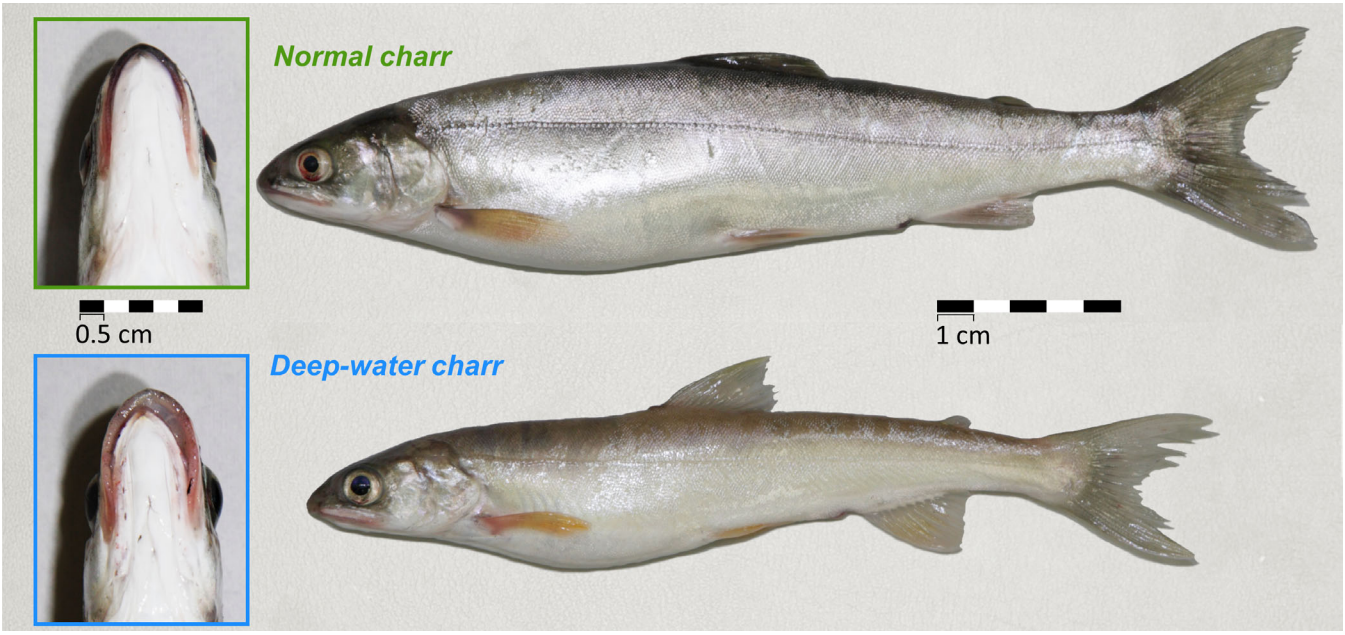

FIGURE 2 Morphology of normal and deep-water charr of Lake Constance. Insets to the left illustrate the difference in lower jaw position in the two forms. Photo credits: J. Baer.

Neither of these attributes are present in the normal form, which exhibits a subterminal mouth, and a lower jaw that cannot be fitted within the upper jaw (Figure 2). In our study, only four captured individuals could not be confidently assigned to one or other form. Thirty five percentage ($n = 127$) of all caught charr belonged to the deep-water form (Appendix S4: Table S1). Deep water charr were captured at sampling locations 2 and 3 in every fishing campaign, but never at location 1 (Appendix S4: Table S1). Normal charr were caught at nearly all sampling dates and locations (Appendix S4: Table S1).

All normal charr captured during surveys in November and December exhibited TL > 250 mm and were sexually mature. Females and males had mean GSIs of 19.4% ± 3.0% (mean ± standard deviation [SD]), and 3.5% ± 0.9% SD, respectively. All these mature individuals belonged to age classes of 4 years or older. None of the deep-water charr captured during autumn and winter was sexually mature, and highest GSI-values were found in individuals caught during July and August with mean values of 18.2% ± 2.5% for females and 4.2% ± 1.2% for males. As with normal charr, mature deep-water individuals were all allocated to age class 4 or older. A very few deep-water individuals caught in August had already spawned, whereas all September captures had spawned ($n = 8$).

The stomachs of 56% ($n = 25$) of analyzed normal charr and 68% ($n = 25$) of deep-water charr contained prey. In the contemporary samplings the main food source for normal charr appeared to be pelagic zooplankton, a marked difference to 1972 when stomach contents were dominated by chironomids (85%) and pelagic zooplankton amounted to just 15% (Figure 3). Contemporary deep-water charr appear to be feeding mainly on turbellarians (Figure 3), just as they did in 1972, although there were differences in the proportions of other dietary components, with historical samples containing fewer

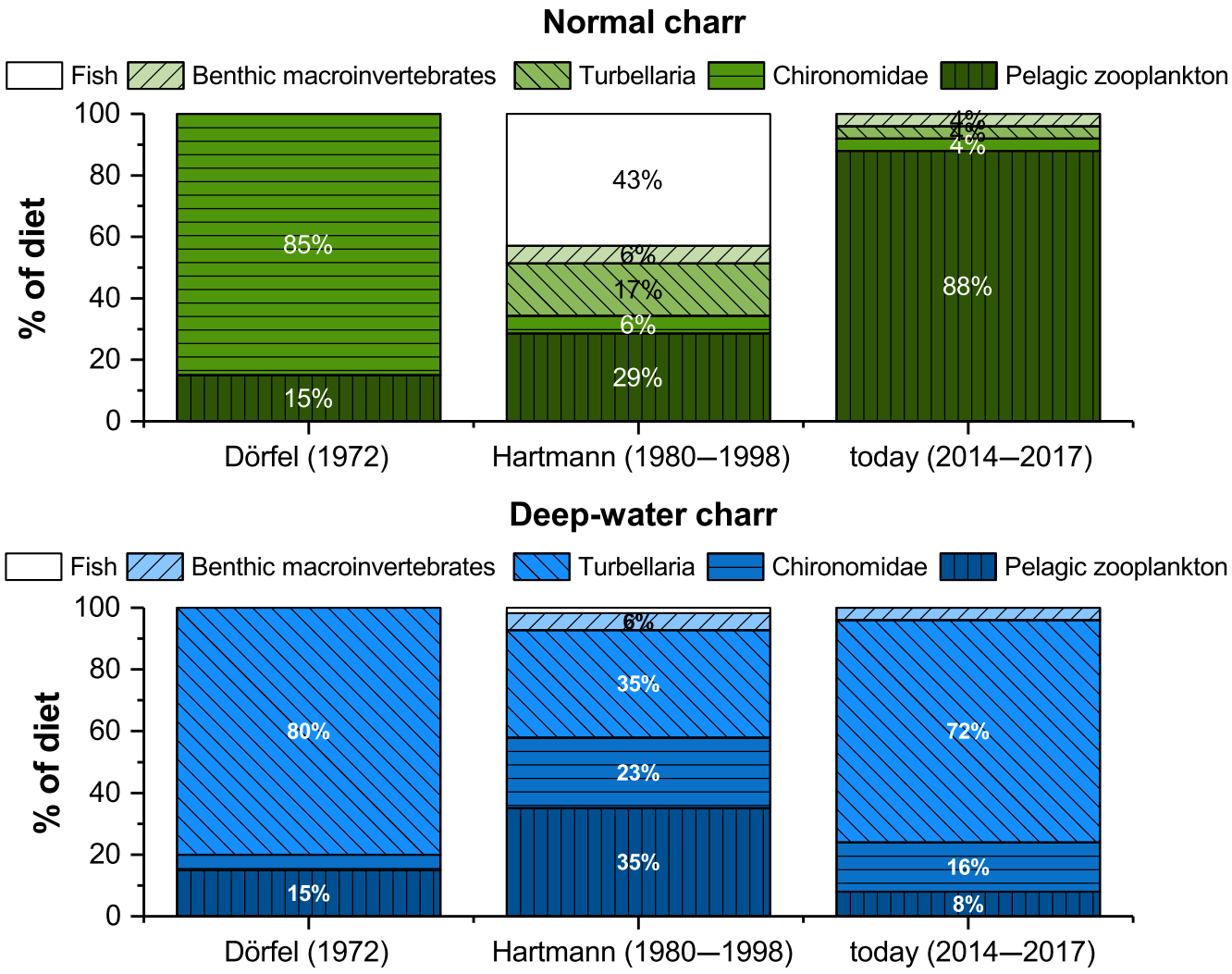

FIGURE 3 Relative occurrence of prey category in diets of normal and deep-water charr forms in the samplings of Dörfel (1974), Hartmann (1980–1998) and today.

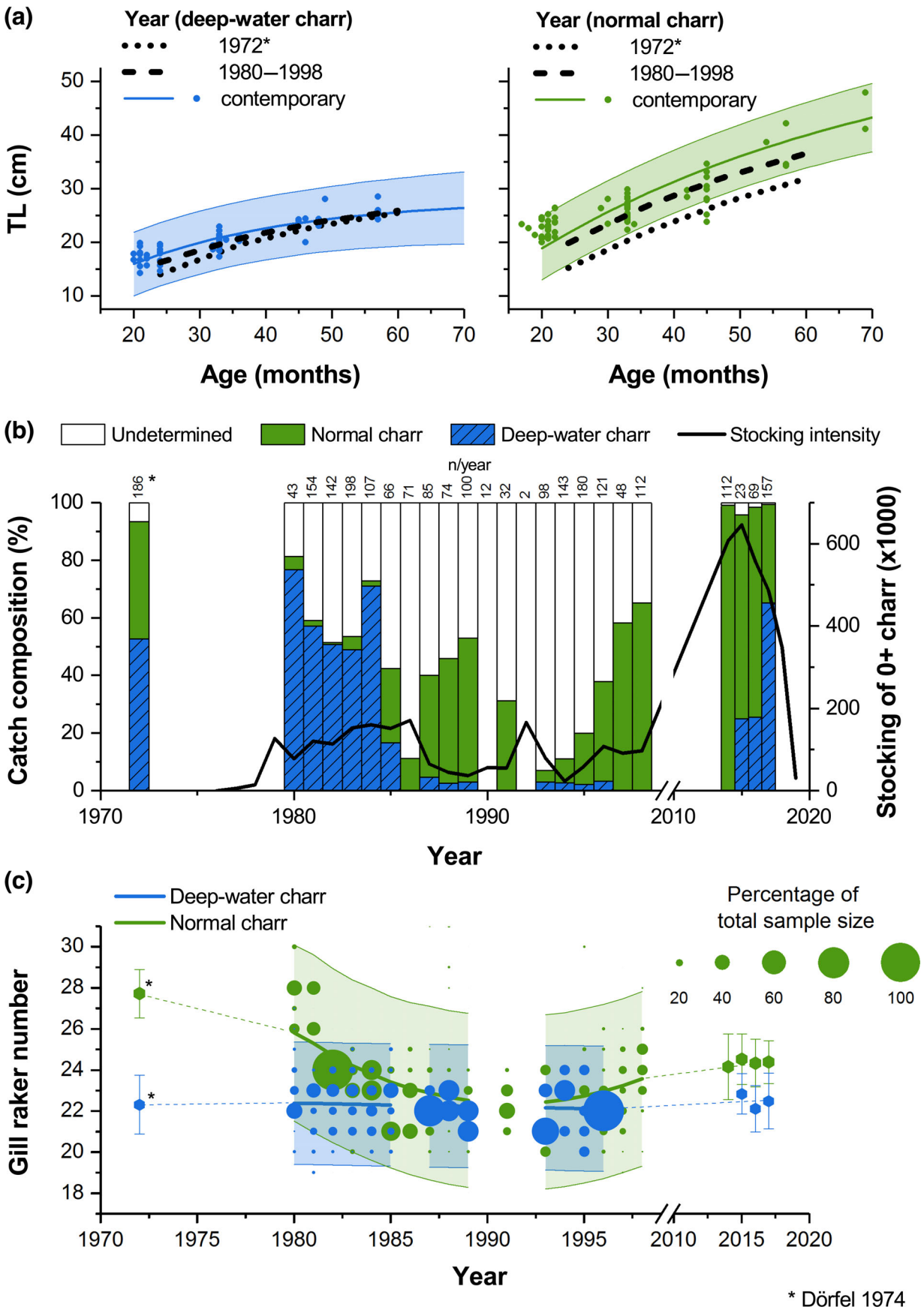

FIGURE 4 Legend on next page.

chironomids (5% compared to 16% today) and more pelagic zooplankton (15% compared to 8% today) (Figure 3). Thus, the diet for both forms appears to have changed over time (Cochran–Mantel–Haenszel test, $p < 0.0001$, $\chi^2 = 200.99$, df = 4).

Age determination of the deep-water charr was problematic, being impossible for 48.1% ($n = 61$) of all captured specimens, due to the absence of distinct hyaline zones in both otoliths and scales. Age determination in the normal form by scales was possible for 161 individuals (69.0%). Of those that could be aged, no age class 1 (0+) individuals were recorded for either form. The oldest, reliably determinable individuals were from age class 6 (normal charr) and age class 5 (deep-water charr). Growth models revealed greater asymptotic growth in normal charr than in deep-water charr (Figure 4a). The von Bertalanffy growth models (Figure 4a) both assume group-specific asymptotic length (King, 2013). For normal charr, the body growth coefficient (K) was 0.26, asymptotic length (L_∞) was 514 mm, and age at which length is zero (t_0) was 0.04. For deep-water charr, the corresponding values were $K = 0.39$, $L_\infty = 299$ mm, and $t_0 = 0$. Growth curves from the 1972 data did not differ significantly from those of contemporary deep-water charr, but indicated unequivocally slower growth compared to contemporary normal charr (Figure 4a).

Data from 1980–1998

Of the 1822 charr sampled by Hartmann between 1980 and 1998, 757 individuals (41.5%) could be assigned to one of the two forms: 396 (21.7%) were classified as deep-water charr, 361 (19.8%) as normal charr (Figure 4b). The remaining 1065 specimens (58.5%) remained indeterminable. Most deep-water charr (97.4%, $n = 339$) were collected in the years 1980 to 1985 (Figure 4b), just 2.6% ($n = 22$) were found between 1986 and 1996, and not a single individual was recorded after 1996. Between 1980 and 1984 normal charr were underrepresented in the catch composition, only exceeding 20% of yearly catch from 1985 on (Figure 4b). In the 1990s, the proportion of normal charr in the catch increased and peaked at more than 50% in the last 2 years of Hartmann's records (1997–1998) (Figure 4b).

One hundred and forty of the charr that could be assigned to one or other forms (119 deep-water charr and 25 normal charr) had been aged by Hartmann from scales. The oldest of these individuals were from age class 5. Growth models revealed significantly greater asymptotic growth for normal charr than for the deep-water form (Figure 4a). For normal charr, the body growth coefficient K was 0.24, $L_\infty = 517$ mm, and $t_0 = 0.04$. For deep-water charr, the corresponding values were $K = 0.38$, $L_\infty = 302$ mm, and $t_0 = 0$. For deep-water charr, growth curves were highly comparable with those of contemporary and 1972 specimens (Figure 4a), but those of Hartmann's normal charr exhibited faster growth than recorded in the data from Dörfel (1974) but slower than today (Figure 4a).

Hartmann noted the main food items found in the stomach of 320 charr that could be assigned to one of the two forms (285 deep-water and 35 normal specimens) and comparison of these data with older and contemporary sampling reveals strong dietary differences over time among normal charr (Figure 3). The most striking outcome is the high percentage of fish (43%) in the diet of normal charr recorded by Hartmann (Figure 3) which was not the case in either 1972 or in contemporary samplings. For deep-water charr, the differences were less apparent, however, during the era of Hartmann (1980–1998) samples yielded considerably higher percentages of pelagic zooplankton and fewer turbellarians (Figure 3). Thus, it appears that charr diet has varied significantly both between forms and over time (Cochran–Mantel–Haenszel test, $p < 0.0001$, $\chi^2 = 162.41$, df = 4).

Stocking and development of gill raker number

Intensive stocking, most likely with *S. umbla*, began in Lake Constance after the Second World War, with 4000 Scottish charr (TL 70–90 mm) introduced in 1977, 6000 charr from different lakes in Bavaria and Switzerland (TL 120–150 mm) in 1978, and 15,000 charr (TL 60–100 mm) from Bavaria and Scandinavia in 1979.

FIGURE 4 Differences between the two charr forms in Lake Constance. (a) Mean length of both charr forms at certain age classes. Solid lines are von Bertalanffy growth curves, toned areas represent upper and lower 95% confidence intervals, dashed lines are historical growth curves in 1972 and between 1980 and 1998. (b) Catch composition during historical sampling in 1972, between 1980 and 1998 and during contemporary sampling between 2014 and 2017. Solid lines indicate yearly stocking intensity with 0+-charr. (c) Mean gill raker number of both charr forms from 1972 (bars indicating standard deviation), gill raker number as a percentage per year per form between 1980 and 1998 (toned areas represent upper and lower 95% confidence intervals) and mean gill raker numbers with standard deviation (error bars) for both forms from contemporary sampling between 2014 and 2017. TL, total length.

In 1980, more than 100,000 charr (TL around 100 mm) from Switzerland were stocked, and in the years between 1981 and 1998 a total of 1.9 million charr of varying provenances were released in the lake in cohorts of up to 200,000 individuals in a given year (Figure 4b). In 1987, a spawner stock was established at the Fish Hatchery Station of Baden-Württemberg, Germany, using adult charr caught in the lake (E. Dossow, personal communication, Fischbrutanstalt Langenargen, Germany), and fish released from 1991 onward came from this stock. Between 1996 and 2015, an average of 351,000 ± 204,000 (±SD) charr (TL 70–90 mm) were stocked annually (Figure 4b). This program was virtually suspended in 2015 for management reasons, and the stocking intensity between 2016 and 2019 fell below 50,000 charr per year (TL 60–80 mm). The total number of 0+-charr stocked into Lake Constance between 1977 and 2019 was 9.2 million (approximately 230 individuals ha^{-1}, here: into the pelagic area of ULC, see Gugele et al., 2020), at least 2.2 million of them (55 individuals ha^{-1}) of foreign origin.

The gill raker count in normal charr in Lake Constance decreased sharply after 1980 and by the late 80s had fallen below the number observed in historical descriptions of the form (Figure 4c) so that, by the end of that decade, gill raker counts of normal and deep-water charr were almost the same (Figure 4c). However, the counts in normal charr began to increase again slowly from the mid-1990s (Figure 4c). The GLM ($r^2_{\text{adjusted}} = 0.07$, $n = 357$, $p < 0.0001$) analysis reveals a highly significant negative correlation ($p = 0.0002$) between stocking intensity and gill raker number over the period 1980 and 1998. The interaction of stocking and phosphorus concentration also shows a significant negative correlation ($p = 0.048$) with gill raker number. No significant impact was detected for any of the other tested parameters.

Morphological differences

The deep-water charr from contemporary samples had a mean TL of 199 ± 28 mm (mean ± SD), with the smallest individual measuring 124 mm and the largest 302 mm (Table 1). Normal charr were larger on average, at 274 ± 49 mm ranging from 164 to 479 mm. Mean body masses for deep-water and normal forms were 52 ± 24 and 164 ± 125 g, respectively. The gill raker counts of normal charr were significantly higher than those of deep-water charr at 24.2 ± 1.4 SD and 22.4 ± 1.3 SD, respectively (*t*-test, $p < 0.05$). The gill raker counts of contemporary deep-water charr were not significantly different (*t*-test, $p = 0.36$) from those recorded in historical data from the years before intensive stocking began (22.3 ± 1.4 SD, Dörfel, 1974), whereas those of the endemic normal specimens recorded by Dörfel (1974) were distinctly higher than today, at 27.7 ± 1.2 SD (*t*-test, $p < 0.0001$).

TABLE 1 F-statistics (fixation indices) as a measure of pairwise population differentiation for four and two populations, respectively, mean Fst is based on one randomly selected SNP per locus (7566 and 7771, respectively), and mean Fst' and mean Phi st are Fst-analogs for haplotypic data of these loci.

Population comparisons	Mean Fst	Mean Phi st	Mean Fst'
Four populations comparison			
Fließhorn deep-water versus Fließhorn normal	0.100	0.135	0.095
Fließhorn deep-water versus Arbon normal	0.094	0.130	0.092
Arbon deep-water versus Fließhorn normal	0.104	0.144	0.098
Arbon deep-water versus Arbon normal	0.098	0.137	0.095
Fließhorn deep-water versus Arbon deep-water	0.021	0.013	0.007
Fließhorn normal versus Arbon normal	0.021	0.012	0.007
Two populations comparison			
Deep-water versus normal	0.110	0.168	0.141

Note: All values were calculated in the populations-tool as described in the stacks manual (Catchen et al., 2013). Sample sizes are *N* Fließhorn deep-water = 19, *N* Arbon deep-water = 24, *N* Fließhorn normal = 18, *N* Arbon normal = 23.

The GLM for ED ($r^2_{\text{adjusted}} = 0.66$, $n = 361$, $p < 0.0001$) shows positive correlation with both TL and size-corrected HL, both of which exert highly significant effects on ED ($p < 0.0001$). Sex has also a small but significant effect ($p < 0.0001$) with females possessing slightly larger eyes than males of the same length (Tukey-HSD, $p < 0.05$). Neither form (deep-water or normal) nor the interaction between HL and TL had an influence on ED.

The statistical analysis of HL (GLM, $r^2_{\text{adjusted}} = 0.96$, $n = 361$, $p < 0.0001$) revealed significant influences of TL ($p < 0.0001$), sex ($p = 0.023$), and form ($p = 0.0019$). Post hoc comparisons (Tukey-HSD, $p < 0.05$) showed that the size-corrected HL for male and female deep-water charr (43.1 mm ± 1.6 SD and 41.7 mm ± 1.6 SD, respectively) were significantly larger than for male and female normal charr (41.9 mm ± 1.5 SD and 39.9 mm ± 1.3 SD). The interaction between sex and form showed no significant influence on HL.

The GMM comparing contemporary and historical samples of both charr forms identified distinct differences in body shape. The results of the PCA are shown in Figure 5b. The first two PC account for 51% of variance

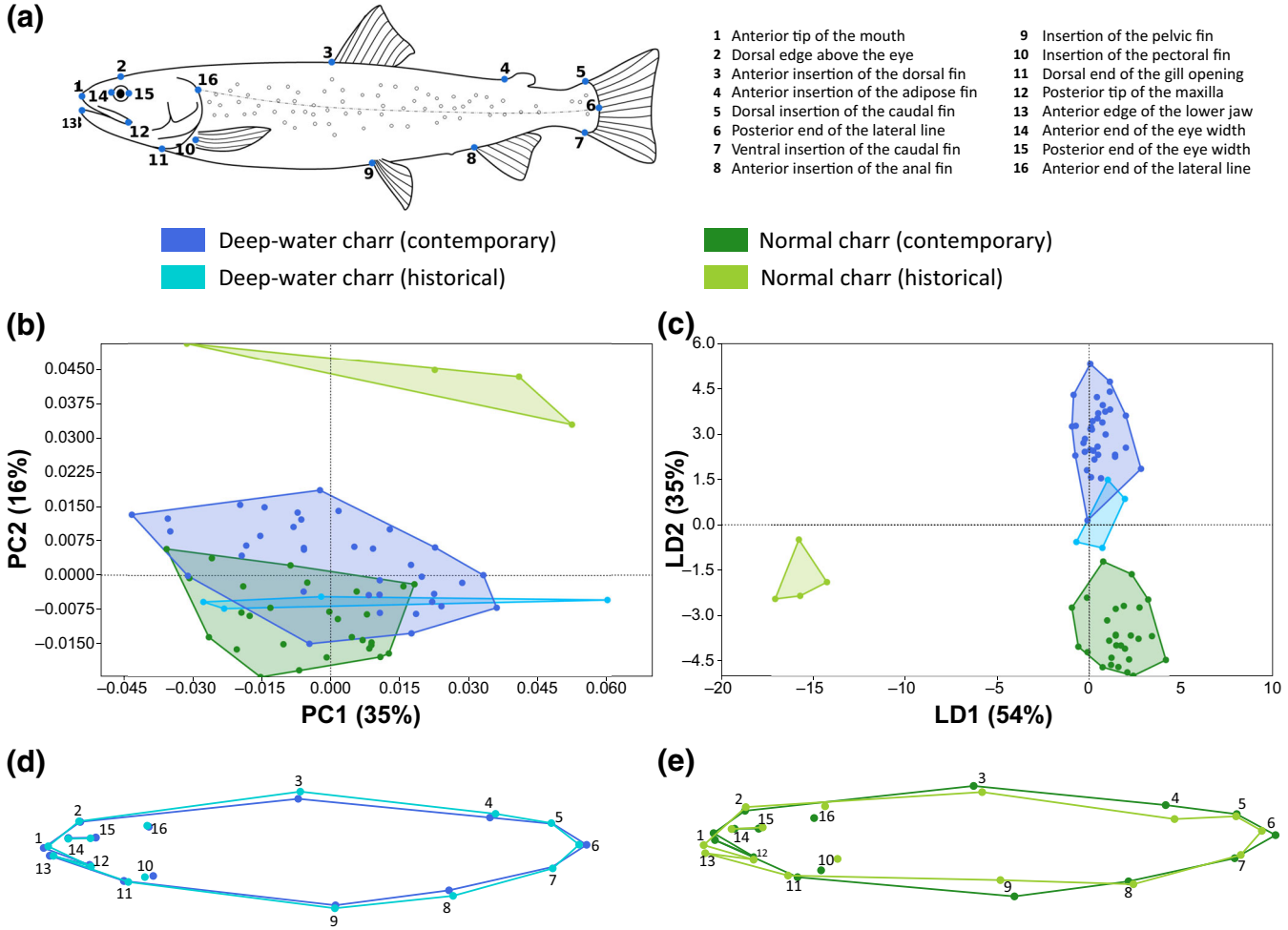

FIGURE 5 Results of the geometric morphometric analyses of contemporary and historical deep-water and normal charr samples. (a) Overview and description of morphological landmarks used in the analysis. (b) Results of the principal component analysis, showing first two principal components (PC), which explain most of the variance in the data (see axis labeling in percent). (c) Results of the linear discriminant analysis, showing the first two linear discriminants (LD) that explain most of the variance in the data (see axis labeling in percent). (d) Wireframe graph of average shape differences between historical and contemporary deep-water charr. (e) Wireframe graph of average shape differences between historical and contemporary normal charr.

in the data. The results of the LDA showed a separation of individuals into four morphometric groups (Figure 5c), where the first two discriminant variables described 89% of variance in the data. The Jackknife cross-validation allocated 93% of all individuals correctly. Ignoring a priori knowledge of the historical samples (form assignment) from the LDA and building only two groups (normal and deep-water charr), the Jackknife cross-validation assigned only one historical specimen to the contemporary normal form. Finally, the DFA revealed no statistically significant differences between the Procrustes distances of historical and contemporary deep-water charr ($p = 0.0887$, Figure 5d) and the wireframe graphs of contemporary and historical samples were highly congruent (Figure 5d). In contrast, the Procrustes distances between historical and contemporary normal charr were significantly different ($p = 0.0001$, Figure 5e), with recent forms exhibiting a more erect, terminal mouth and slightly greater body depth (distance between insertion of the dorsal and pelvic fin) (Figure 5e).

Genetic analysis

After filtering of the data, 59,758 RAD loci of sufficient data quality were retained for population genomic analysis of 84 contemporary charr individuals, which had been assigned a priori to four groups by phenotype (normal and deep-water) and sample location ("Fließhorn" and "Arbon"). Among these, 7566 loci had variant sites available for coancestry analysis in fineRADstructure. The two population analysis, in which individuals were

grouped according to phenotype only (normal vs. deep-water), yielded 62,112 RAD loci, of which 7771 SNPs were randomly retained. Pairwise divergence Fixation index statistics (F-statistics) indicated a substantially stronger separation between deep-water and normal charr populations than between samples of the same phenotype (Table 1).

Calculation of additional population descriptive statistics in contemporary samples revealed a substantially higher private allele count and slightly higher values for heterozygosity in normal charr than in deep-water charr (Appendix S5: Tables S1–S3).

The fineRADstructure coancestry analysis based on the 7566 SNPs dataset revealed strong population structure differentiation between contemporary deep-water and normal charr forms (Figure 6). Notable exceptions were three individuals classified phenotypically as normal charr, which showed approximately equal shared coancestry with both the deep-water and the normal form. These specimens are hereafter referred to as the "intermediate form" (Figure 6). Otherwise, intra-phenotypic coancestry differentiation appears comparatively weak, with deep-water charr showing two main clusters. All but one of the specimens in the smaller cluster were from sampling location 2, Fließhorn, while members of the larger cluster derived from both locations. Two individuals were highly similar, possibly indicating sibling relationship (Figure 6).

A population genetic analysis of 89 diagnostic SNPs selected a priori on the basis of high locus-specific Fst-values between contemporary charr forms (normal and deep-water) (see *Material and Methods*) was performed to assess genetic similarity and tentative population assignment of the 12 historical scale samples with extant specimens. The PCoA revealed strong differentiation between two major clusters on PC1, which explained 24.1% of variance in the data set, whereas PC2 accounted for only 3.4% of variance (Figure 7a). As expected from the a priori bait-locus selection, the two clusters showed significant differentiation between the extant forms on PC1 (Figure 7a). Eleven out of twelve historical samples (assigned a priori to normal or deep water forms) fell unambiguously within the deep-water charr cluster (Figure 7a). Only one historical sample plotted at an intermediate position outside the extant normal charr cluster (Figure 7a). The three extant individuals exhibiting mixed normal and deep-water ancestry in the fineRADstructure analysis (see Figure 6) grouped on the margins of the extant charr cluster and close to the single anomalous historical sample (Figure 7a). A Jackknife cross-validation between the three normal charr in the intermediate group and other extant normal charr showed no morphometric differences (Figure 5b), but highlighted significantly higher gill raker numbers in the intermediate form (t-test, $p < 0.05$).

The PCoA result is supported by the results of the Bayesian population assignment of the 12 historical samples using *STRUCTURE* (Pritchard et al., 2000). The ΔK method had identified the most likely number of clusters present in the 89 diagnostic SNPs data set as two ($K = 2$ mean value of ln likelihood −4629.0; $K = 3$ mean value of ln likelihood −4502.0). All historical samples were assigned with proportions higher than 92.4% to the extant deep-water charr type except for the one historical normal charr specimen which plotted in the PCoA analysis at an intermediate position with only 53.1%, and one historical deep-water charr plotted within the deep water charr cluster with only 77.5% assignment proportion (Figure 7b). Furthermore, when assuming two genetic clusters ($K = 2$), the three extant intermediate individuals showed slightly increased estimated assignment proportions to deep-water charr (15.2%, 33.7% and 35.7%), approaching the 53.1% of the single historical normal charr with an intermediate position in the PCoA. The $K = 2$ *STRUCTURE*-analysis revealed additional five extant normal charr individuals with spurious ancestry proportions of deep-water charr (i.e., from 3.3% and 11.1%) and an additional 3% historical samples with spurious ancestry proportions of extant normal charr of between 2.8% and 7.5%. All other charr individuals were classified with assignment proportions higher than 98% to either of the two clusters. An explorative structure-assignment of all extant and historical samples assuming three ($K = 3$) rather than two ($K = 2$) population clusters did not assign all intermediate individuals to a distinctive third cluster, but indicated additional clustering within the extant normal charr specimens (Figure 7b). All deep-water charr from contemporary samplings were assigned with 100% to the deep-water charr cluster, whether three ($K = 3$) or two ($K = 2$) population clusters were assumed (Figure 7b).

DISCUSSION

The study presented here demonstrates a timely and effective methodology for describing the genetic status of a rediscovered, but partially known, rare and cryptic living species. Our two-step approach (involving RAD sequencing, and the design of diagnostic RNA baits for target capture of informative loci in DNA from archival scale samples to analyze the degree of divergence to extant samples), provides a blueprint for conservation genomic assessments in future studies where archival samples are required to critically test for levels of extant autochthonous genetic integrity of rediscovered cryptic

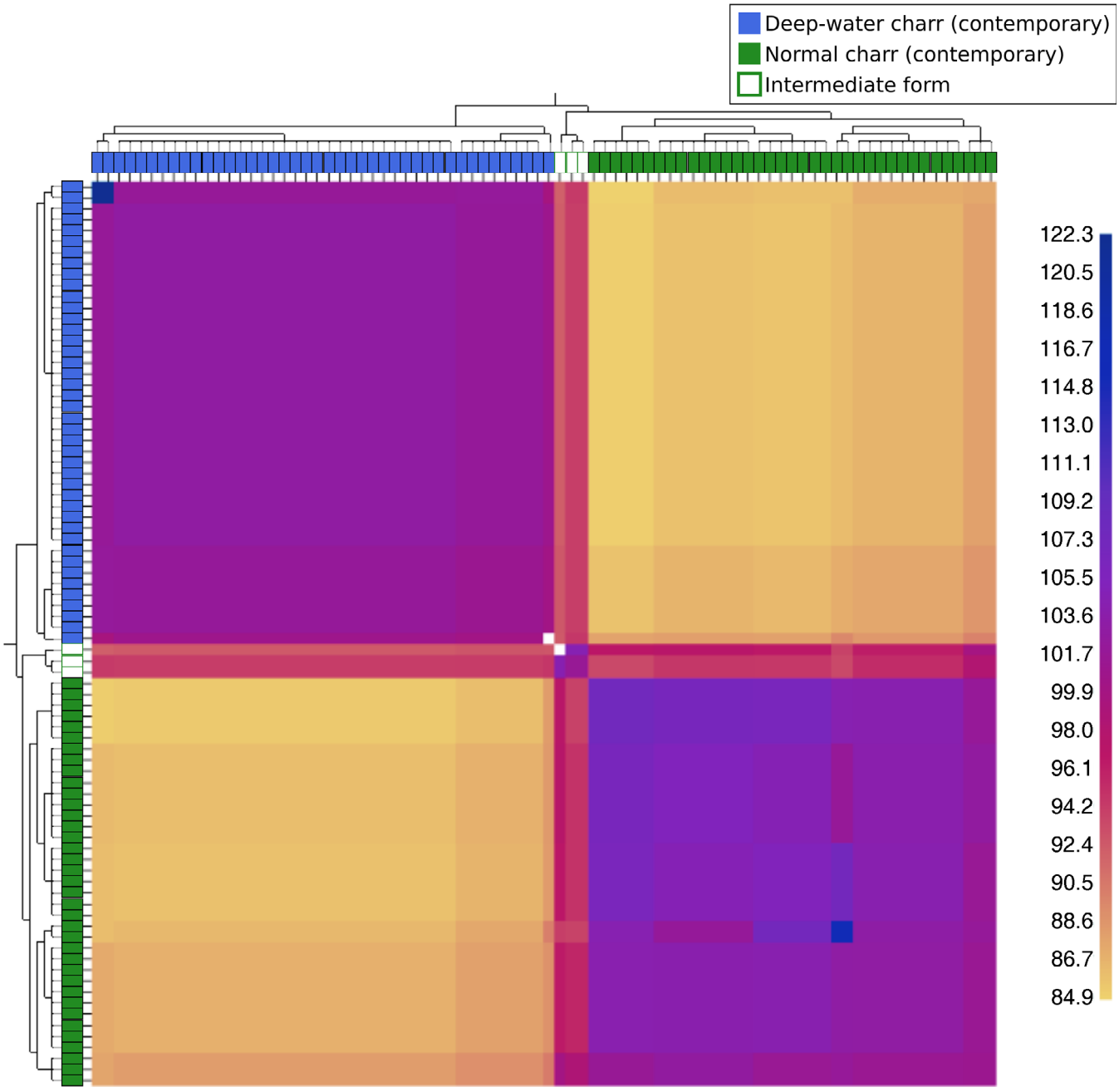

FIGURE 6 Structure of extant charr population in Lake Constance as inferred by a Bayesian clustering approach grouping specimen restriction site associated DNA (RAD)-derived haplotype data according to nearest neighbor coancestry, calculated with the software package fineRADstructure (Malinsky et al., 2018). The depicted coancestry heatmap illustrates degree of pairwise coancestry, that is, level of nearest neighbor haplotype relatedness between specimens, with yellow representing lowest, orange and red intermediate, and purple and blue indicating greatest degree of pairwise coancestry. The dendrograms ("trees") are based on estimated relationships between population clusters. The fineRADstructure analysis indicated two major population clusters with shared high coancestry, consistent with deep-water (blue squares, $n = 42$) and normal charr (green squares; $n = 37$) populations. It also supports the distinction of three specimens (open green squares), which presented phenotypically as normal charr, into a group intermediate between the two major clusters.

taxa. This approach may overcome some of the problems likely to be encountered at the time of rediscovery of extinct believed species (e.g., missing data at GenBank, scarcity of museum samples). Furthermore, by incorporating genetic, morphological, and ecological traits, our approach may provide valuable reference points for other researchers encountering similar problems such as uncertainty over the impact of stocking on a rediscovered species. The co-availability of historical specimen samples and scientific descriptions alongside extensive contemporary sampling data made for a clear course of action after the rediscovery of Lake Constance deep-water charr, but

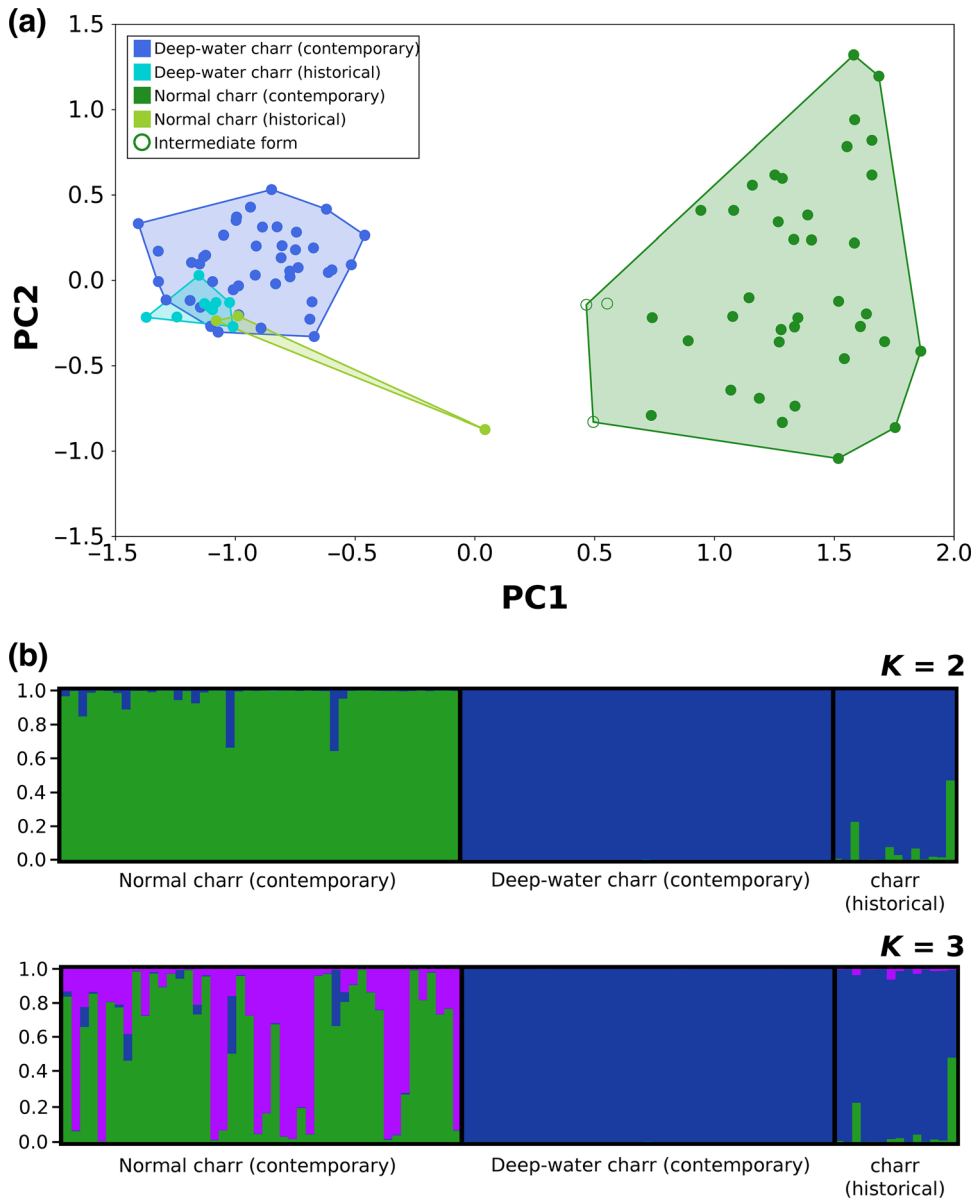

FIGURE 7 Results of population structure analyses based on 89 SNPs assessed by independent restriction site associated DNA (RAD)-analysis as diagnostic for either the extant normal or the extant deep-water forms of charr. (a) Genetic structure of 83 extant and 12 historical *Salvelinus* samples from Lake Constance, as inferred by a Nei-genetic distance-based principal coordinates analysis (PCoA). Displayed are the first and second principle component. (b) *STRUCTURE* barplots (Pritchard et al., 2000) visualizing proportions of ancestry (*y*-axis) for each genotyped individual (*x*-axis) assuming either two (above; $K = 2$) or three (below; $K = 3$) populations; $K = 2$ is the number of clusters supported by the ΔK method for this data-set (Evanno et al., 2005). SNP, single nucleotide polymorphism.

the situation is unlikely to be unique. By deploying a broad range of different methods to describe the genetic, morphometric, and ecological status of both forms we can now show: (I) that the extant normal form of Arctic charr likely represents a population of hybrid origin formed as a consequence of eutrophication stress and intensive stocking, while the endemic normal form is likely extinct, with only marginal evidence for the persistence of autochthonous genomic elements in the extant genepool; (II) that extant Arctic charr in Lake Constance represent two phenotypically and genomically distinct populations; and (III) that morphologically and genetically distinct deep-water charr have persisted in Lake Constance largely unaffected by anthropogenic impact (Figure 8).

The results presented here support the hypothesis that the extant normal charr of Lake Constance are of predominantly allochthonous origin and do not correspond to the endemic phenotype (Table 2). This conclusion is supported by our genetic approach and the fact that not a single SNP-genotype from 12 historical samples fell into the genomic cluster as the extant normal charr

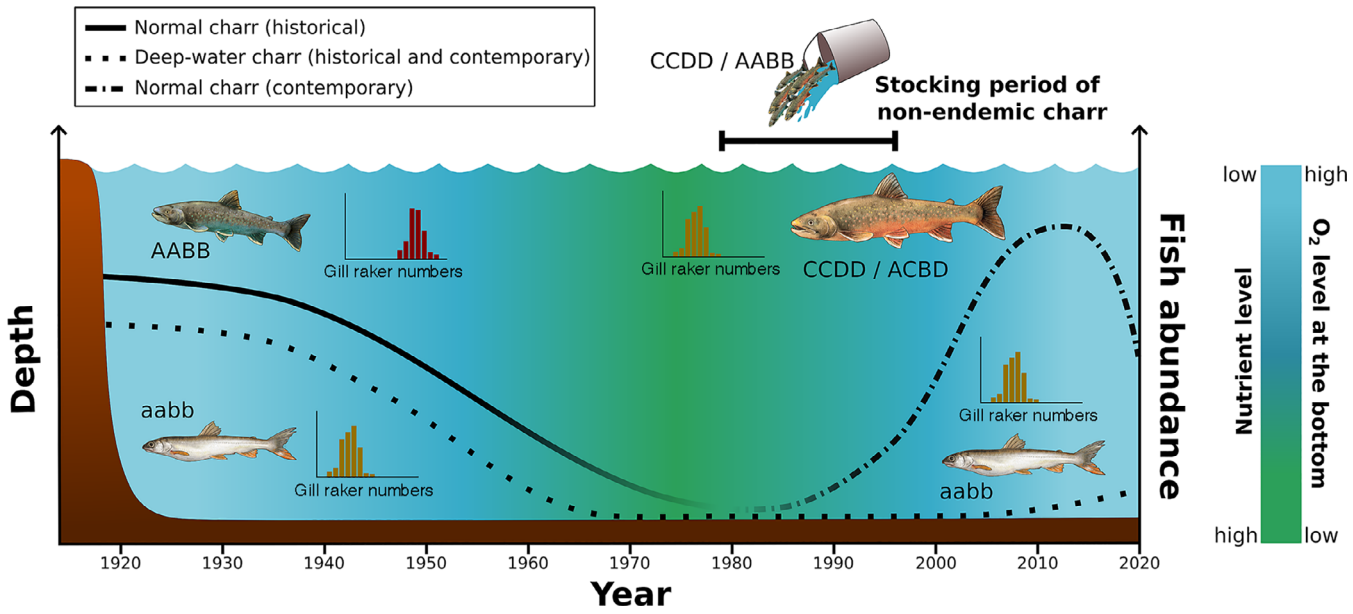

FIGURE 8 Proposed scenario of charr genetic and morphological (gill raker) stock development juxtaposed with anthropogenic stress factors. A dramatic decline in both endemic charr forms in Lake Constance between the mid-1970s and late 1990s appears to be linked to eutrophication and its oxygen implications. Stocks of the deep-water form recovered largely unaltered after re-oligotrophication of the lake, while the endemic normal charr most probably went extinct and was replaced or at least massively introgressed by stocked allochthonous normal charr.

TABLE 2 Summary of characteristics of contemporary and historical deep-water and normal charr.

Characteristic	Deep-water historical	Deep-water contemporary	Normal historical	Normal contemporary
Growth	...	Same as historical	...	Faster as historical
Maximum TL (mm)	279	299	441	514
Main diet	Turbellarians	Turbellarians	Chironomids	Pelagic zooplankton
Body shape	...	Same as historical	...	Different to historical
Mouth type	Inferior	Inferior	Inferior	Terminal
Genetic structure	...	Same as historical	...	Different to historical
Mean gill raker number	22.3	22.4	27.7	24.2

genotypes, although a few intermediate individuals showed low levels of shared ancestry with contemporary samples. Even the one historical specimen phenotypically classifiable as normal charr (from 1958), plotted as intermediate between deep-water and extant normal charr in a PCoA and exhibited a genomic co-ancestry with extant normal charr of only about 50%. The two additional intermediate individuals which showed slightly increased levels of co-ancestry and plotted close to this single historical individual are well separated from the extant normal charr cluster. Furthermore, an explorative *STRUCTURE*-assignment of all extant and historical samples assuming three ($K = 3$) rather than two ($K = 2$) population clusters revealed that a high proportion of extant normal charr could be assigned with a up to 90% probability to a third cluster whereas all historical samples exhibited a low likelihood (0%–5%) of belonging to this third cluster. Further evidence for extant normal charr being dominated by stocked individuals is provided by a stark contrast in growth performance compared with historical individuals (the growth curve of historical normal charr is below the lower 95% confidence interval of the growth curve of extant normal charr, see Figure 4), a likely result of stocking with fish cultured from lineages selected for fast growth. The millions of charr stocked into Lake Constance over the years may have had the potential to both displace and hybridize with endemic charr and pass on their faster growth trait (Tiberti & Splendiani, 2019). An alternative hypothesis, that the observed difference in growth in normal charr might reflect a diet shift (Malmquist et al., 1992) toward piscivory (Eloranta et al., 2011, 2015; Klemetsen et al., 2003), has to

be rejected, as the data presented here indicate a switch in feeding preference from macrozoobenthos during the 1970s (Dörfel, 1974) to pelagic zooplankton today. Fish featured in stomach contents only during the period of intensive stocking with charr of foreign origin (1978–1992). Furthermore, our results also show a remarkable contrast in body shape between historically and recently sampled normal charr (Table 2). This difference recorded by LDA is substantially larger than that observed between historical and recent deep-water specimens (see Figure 5). Although shrinkage is a well-known effect of preservation in formalin or ethanol (Nordeide, 2020; Parker, 1963), it can only partly explain the significant differences in shape, as both PCA and DFA point to differences of jaw position rather than the whole body changes normally associated with shrinkage effects: contemporary normal charr possess a terminal mouth while in historical specimens the mouth is distinctly subterminal.

Based on the genetic results presented here, it is difficult to determine whether extant normal charr have completely replaced historical normal charr or whether they represent a mixed, at least partially hybridized assemblage comprising individuals of different ancestry. Our analysis was performed on 89 hybridization capture bait-sequences based on genomic divergence within contemporary Lake Constance charr populations and was thus biased against the detection of divergence between historical deep-water and extant normal charr. In the absence of a large-scale genomic representation SNP dataset for the historical samples, we must assume that confounding effects for the characterization of historical charr genomes exist. However, the clear distinction shown between extant and historical samples remains valid, since our population genetic analysis revealed significant genomic differences between historical and extant samples of normal charr and the population assignment test did not group any of the historical samples with contemporary normal charr. Nevertheless, the genetic clustering of three extant charr specimens classified phenotypically as normal charr at an intermediate position rather close to a historical normal charr sample is interesting. Even if those three intermediate specimens showed no difference in body shape from other normal charr, their gill raker counts were higher than most contemporary normal charr and matched those documented for the normal form 50 years ago (Dörfel, 1974). Thus, it is possible that these three individuals represent a small subsample of normal charr from Lake Constance with autochthonous genomic components, which persisted through the eutrophic and stocking phases of the lake's history (Hartmann, 1984). Future studies comparing this subset with charr populations used for stocking will be necessary to further test this hypothesis. As acknowledged above, the bias in our SNP-panel does not allow us to infer the extent of historical normal charr ancestry in the extant normal charr population. Given the rapid improvement in genotyping methods for archival DNA, future studies should target the retrieval of a fully representative SNP dataset from our archival samples from which the unbiased degree of shared ancestry of extant and historical normal charr can be assessed. Results could then be used to implement a conservation strategy to enhance the extant normal charr population in Lake Constance by using lines rich in ancestral genomic components identified in historical normal charr.

A second main finding of the study is that the two extant coexisting morphotypes of Lake Constance Arctic charr are highly reproductively isolated. The autecology data show that the deep-water form spawns exclusively during summer, while the normal form has a well-defined spawning period in early winter. The lack of overlap in spawning time apparently prevented mixing of the gene pools of stocked normal charr and surviving endemic deep-water charr, as confirmed by our population genomic analysis. Secondary suggestions that uniform conditions in the profundal zone may allow deep-water charr to spawn over a prolonged period between July–February or even at any time of year (Freyhof & Kottelat, 2008) are not supported by either historical (Dörfel, 1974) or contemporary evidence from Lake Constance, in which gravid females were found exclusively in July and August. Furthermore, the known spawning sites differ spatially, with normal charr favoring the site known as "Teufelstisch," where the deep-water form has never been known to spawn (this study, annual charr monitoring, J. Baer, unpublished data, Dörfel, 1974). These temporal and spatial barriers to hybridization explain the resilience of deep-water charr to potential introgression from introduced stock. Nevertheless, the results of our *STRUCTURE*-analysis do show slightly elevated proportions of historical deep-water charr ancestry in historical normal char, and vice versa (Figure 7b). Again, it is necessary to acknowledge the limitations of the 89 SNP-panel in illuminating historical patterns consistent with restricted gene flow between charr forms. These data do not allow us to infer historical levels of restricted gene flow between the two forms, because they are restricted to divergences between the extant forms. The 12 historical samples available are too few to allow testing of patterns consistent with restricted gene flow between historical charr populations, particularly with regard to historical normal charr. Nevertheless, it is worth mentioning that, according to our fineRADstructure coancestry analysis, three individuals morphometrically indistinguishable from other

extant normal charr but featuring significantly elevated gill-raker counts, appear to exhibit mixed coancestry (Figure 6). In contrast, not a single specimen morphometrically classified as deep-water charr showed this genetic intermediacy, which points toward unidirectional gene flow from deep water charr into extant normal charr, but not vice versa. The reasons for this putative unidirectional gene flow are not clear. Normally, hatcheries that produce fish for stocking purposes use almost exclusively winter spawners (Gillet, 1991; Jeuthe et al., 2013), but the possibility that spring- or summer-spawning charr were stocked in former times cannot be excluded. If this were the case, introgressive hybridization with limited gene-flow from deep-water charr to extant normal charr may have taken place. Furthermore, as discussed above, it is possible that individuals of normal charr assigned to two different ancestral groups might represent a small subsample of endemic normal charr from Lake Constance, suggesting that the degree of overlap in spawning times and places between forms may have been higher in historical times than today. To test these possibilities, it will be necessary to obtain more archival samples from both historical charr populations and a more representative SNP-dataset, ideally complemented with genomic data from charr stocks that have been used for stocking Lake Constance. In the event that material held in other museums or scientific institutes currently unknown to us despite a thorough search comes to light, this may be used to enlarge the sample size and address this question in future.

The third main outcome of the present study is the morphometric similarity between extant deep-water charr and descriptions of historical specimens (Table 2). The extant specimens still exhibit the strongly subterminal mouth reported more than 100 years ago from a deep-water population, then largely unaffected by anthropogenic activity (Schillinger, 1901). The sexual dimorphism in HL and growth at age seen in contemporary data is also consistent with that documented almost 50 years ago (Dörfel, 1974). Furthermore, even after a dietary switch to pelagic zooplankton during the eutrophic phase, which might be expected to have driven selection for higher gill raker numbers (Kahilainen et al., 2011), no such change is observed. The reason for this observation is unclear; however, other studies investigating whitefish (*Coregonus* sp.) have indicated that gill raker number can remain stable for up to 24 years in the face of changing food sources and varying levels of human impact (Siwertsson et al., 2012). The hypothesis that adaptation to changing diet may affect the shape rather than the number of the gill rakers, as observed for Artic charr in other lakes (Michaud et al., 2008), could not be tested, because no suitable historical gill arch material was available. Some differences were observed in body shapes of historical, fluid-preserved deep-water charr and recent samples and confirmed by PCA and LDA. However, pairwise comparison indicates that changes over the last 60 years are minor or negligible and all four historical deep-water charr specimens group with the contemporary deep-water samples under a Jackknife cross-validation. Thus, the minor differences in body length and shape are most likely artifacts of fluid preservation. In addition, all DNA-samples derived from the historical scale samples of the deep-water charr can be assigned unambiguously to the extant deep-water charr DNA-type. Furthermore, as discussed above, the unidirectional gene flow from the deep-water form into the normal form reduce the possibility of introgressive hybridization between deep-water charr and stocked fish or even normal charr (Doenz & Seehausen, 2020). A more likely explanation is that the extant deep-water charr in Lake Constance are not morphologically adapted normal charr or hybrid forms, but direct descendants of endemic ancestors which survived the extreme conditions that prevailed during the eutrophic phase of the lake.

The scenario outlined above, namely the replacement of an endemic stock with stocked relatives (here: normal charr) and the driving of closely related sympatric species (here: deep-water charr) to near extinction, was a result of anthropogenic impact. The negative effects of eutrophication in the 1960s and 1970s led to decreasing stocks, and subsequent efforts to reverse this trend by stocking with thousands of charr from foreign provenances compromised the genetic integrity of the normal charr (Figure 8). That the genetic structure of lake-living salmonid populations can be influenced by stocking has been shown in several studies (Lamaze et al., 2012, 2013; Marie et al., 2010; Savary et al., 2017). In Lake Constance, hybridization effects are apparent as increased number of private alleles and a general change in the genetic composition of the extant population of normal charr in the lake. Morphological changes, such as the decrease in gill raker number can also be linked to the stocking of foreign charr. In 1987, 10 years after the intensive stocking began and thus within a reproductive timescale of, at most, three generations, the gill raker number of normal charr in Lake Constance was reduced by approximately 20%. Already, after those few years, the records garnered by Hartmann suggested gill raker number was no longer a valid diagnostic feature for differentiating normal and deep-water charr forms. Divergence in gill raker number is a general pattern in the adaptive radiation of postglacial fish (Østbye et al., 2005), and is often seen to change with emerging foraging strategies in an eco-evolutionary feedback loop (Kahilainen et al., 2011). While eutrophication in Lake Constance led to significant changes in food

sources, including a marked increase in the density of the zooplankton (Straile, 2015), this is deemed an unlikely driver of the observed change in normal charr raker meristics, because the gill raker numbers of other zooplanktivorous fish in the lake, including the pelagic or benthic living whitefish, remained stable (Jacobs et al., 2019), as did those of the deep-water charr. In other pre-alpine lakes where zooplankton density has increased, changes have been observed in the gill raker numbers of zooplanktivorous whitefish (Vonlanthen et al., 2012), but these were generally much smaller in magnitude than seen here in normal charr, and developed over significantly longer time periods (Bittner et al., 2010). Furthermore, in water systems where large shifts in gill raker number have been observed over short timescales (less than 15 years or three to four generations), the driver has been identified as stocking with domesticated genotypes (Dierking et al., 2014; Huuskonen et al., 2017). In light of the short time between stocking and significant changes in gill raker number in Lake Constance normal charr, we consider this is a direct result of stocking and that gene flow between extant deep-water charr and normal charr has been limited in extent.

The data presented here clearly show that effective biological assessment of complex situations like that of Lake Constance charr requires the detailed inspection of multiple data sets. This diligence becomes even more critical where the conclusions are likely to form the basis of conservation actions. In the present case, it appears that stocking offers the most likely explanation for the observed morphological and genetic traits in extant populations of Lake Constance charr, and not introgressive hybridization between endemic forms or rapid adaptation after re-oligotrophication. Many studies of Arctic charr in other lakes have identified stocking as a relevant factor driving change in the genomic biodiversity of endemic populations (Brunner et al., 1998; Savary et al., 2017; Tiberti & Splendiani, 2019) and stocking with allochthonous fish has been common practice in most pre-alpine and alpine lakes for over 100 years (Englbrecht et al., 2002). Conservation practitioners must often act without full knowledge of the factors at play in a given setting but inferences about the integrity of endemic species based solely on recent genetic data without accounting for possible confounding factors like stocking (Vonlanthen et al., 2012) may lead to a false presumption, for example, that the endemic normal charr of Lake Constance still exist in large numbers. The results presented here emphasize that conservation practitioners and policy makers need to be informed about all known and possible population stressors for effective and targeted decision-making in conservation management (Côté et al., 2016).

From our perspective, the three main findings of this study suggest that existing conservation designations of both charr forms in Lake Constance does not reflect the population picture established here. Firstly, we suggest a revision of the existing IUCN status of "Least Concern" for normal charr (evaluated as *S. umbla*, Freyhof & Kottelat, 2008). Based on results of this study and with regard to the low degree of shared ancestry between extant and historical normal charr, we suggest that a classification of "Data Deficient" (DD) is more fitting for this population. Secondly, the data presented in this study show that the deep-water charr, referred to as *S. profundus* and classified as "Extinct" (EX) by Freyhof and Kottelat (2008), are not distinguishable from the historical endemic form and are nowadays caught regularly. We therefore propose re-classification of the deep-water charr (here: *S. profundus*) actually as "Critically Endangered" (CR). The less serious ranking "Endangered" (EN) would contradict IUCN guidelines given the limited data about range and standing stock (IUCN Standards and Petitions Committee, 2019). It is promising however, that spawners are caught regularly during summer, indicating that the population, while very small, is at least stable and reproductively healthy.

To compare growth at age during different time periods, future research will need to develop robust and accurate techniques for age determination, in particular of deep-water charr, which have thus far resisted reliable interrogation using classical age-determining tools and methods. This is a familiar problem in studies of slow-growing fish living in deep-water habitats (Swan & Gordon, 2001; Treble et al., 2008). In the current study, around 50% of individual specimens were of indeterminate age, independent of the tissue used (otoliths or scales) for age determination. The same outcome was reported by Dörfel (1974) and other studies encountered problems aging charr older than 5 years (Frost, 1978). In contrast, Doenz and Seehausen (2020) did not report difficulties and presented age data for 100% of deep-water charr specimens using standard otolith readings. How this precision was achieved is unclear. All deep-water charr described in the current study were caught at depths of 80–100 m, where the water temperature is comparatively stable during the course of the year (4–5°C) and benthic food is always abundant (Gergs et al., 2011; Huber et al., 2011). These factors can even out season variation in growth (i.e., winter and summer), resulting in non-differential growth in structures such as scales and otoliths, making precise age reading more difficult and underlining the need for new and more robust methods.

Future studies should also tackle the question of whether and to what extent stocking practices are responsible for established morphometric differences between species or forms. In Lake Constance, differences in body shape or HL between the two forms of charr have been known for more than 100 years (Schillinger, 1901). The present study shows that some of those differences (e.g., HL) are still apparent, but, given the fact that extant normal charr are neither genetically nor morphometrically comparable with historical ones, such comparisons are no longer valid features for distinguishing charr forms. For example, allometry-corrected ED was not dependent on form. It is therefore questionable how the idea that deep-water charr possess larger eyes than normal charr as a result of adaptation to low light conditions in their deep-water habitat, suggested by Fishelson et al. (2004) and Doenz and Seehausen (2020) might be tested using contemporary samples. The results of the current study indicate that contemporary forms do not differ significantly in ED and only a sufficient sample size of historical samples could answer whether those differences ever really existed.

In summary, the present study presents a globally applicable blended methodology for appraising the genetic and morphological integrity of rediscovered species, and confirms that the dwarf deep-water form of the Arctic charr, described as *S. profundus*, and presumed extinct, still exists in Lake Constance, having survived a period of eutrophication and intense stocking mostly unchanged by virtue of spatially and temporally isolated spawning behavior. In contrast, the integrity of the pelagic extant normal form of Lake Constance charr, *S.* cf. *umbla*, has most likely been severely compromised by stocking, such that contemporary samples now differ significantly from endemic ancestors. We recommend that both forms to be re-evaluated with regard to their conservation status and protection. In addition, this study highlights the importance of considering stocking impacts on fish fauna when working to preserve and foster communities of endangered and cryptic living fish.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Morphometric data (Baer et al., 2022) are available in Figshare at https://doi.org/10.6084/m9.figshare.21102847. Genetic data (Schliewen et al., 2022) are available in Dryad at https://doi.org/10.5061/dryad.41ns1rnhx.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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