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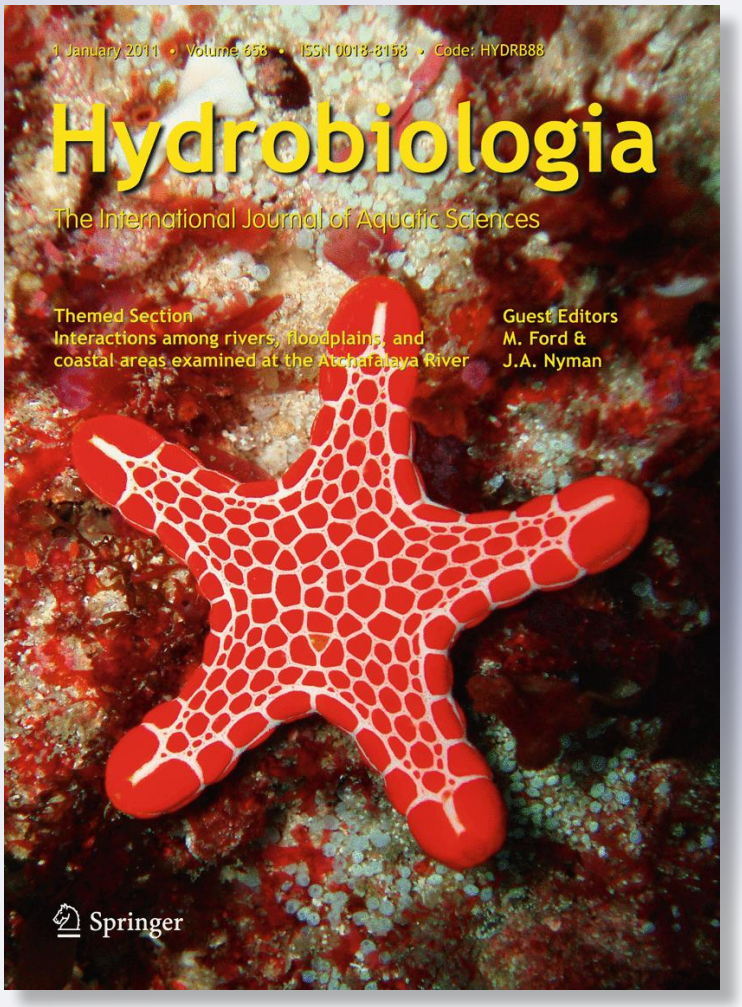

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PRIMARY RESEARCH PAPER

Regional structure despite limited mtDNA sequence diversity found in the endangered Huchen, *Hucho hucho* (Linnaeus, 1758)

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Abstract We evaluate the hypothesis of no geographic structure in the Huchen (*Hucho hucho*), a large, predatory salmonid endemic to the Danube basin. Forty-seven individuals sampled from throughout the Huchen's native range were genetically characterized. Extremely limited sequence diversity across 1,800 bases of mtDNA (the complete control region and partial NADH-1 subunit) evidenced by four closely related mtDNA haplotypes was found. Nonetheless, the geographic distribution of mtDNA repeats (5–10, 82-bp long copies per individual) as well as allelic diversity across two microsatellite loci indicated large-scale geographic structure between the north-western (Austria and Slovenia) distribution area and eastern (Slovakia and Ukraine) or southern (Bosnia-Herzegovina and Montenegro) sample sites. An extremely slow rate of substitution for the *H. hucho* mtDNA is considered along with the alternative hypotheses to explain the limited mtDNA diversity. Considering the regional genetic structure implied by our data, we advocate restrictions on the transport of brood fish or yearlings across the range of the species distribution and sale of Huchen across international boundaries. Future genetic analysis to support local conservation and monitoring efforts must focus on developing a high-resolution screen that may be applied to identify hatchery versus naturally reproduced individuals in the wild.

Keywords Danube salmon · Danubian basin · Phylogeography · Microsatellites · mtDNA

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Introduction

Huchen *Hucho hucho* (Linnaeus, 1758) also known as Danube salmon is one of the most endangered members of the family Salmonidae (Holčik et al., 1988). They are endemic to the Danube basin and historically were common over large areas of the drainage (Holčik 1995). *H. hucho*, along with its sister species taimen *Hucho taimen* (Pallas, 1773), are obligate freshwater residents and nonetheless the largest of all salmonid fishes. While the taimen may exceed 100 kg (Holčik et al., 1988), the largest reported Huchen was 60 kg, caught in the Danube River in 1873, whereas specimens up to 50 kg were still being reported in the mid-twentieth century

(Holčik et al., 1988). Presently, Huchen over 20 kg are noteworthy, but a specimen of 34 kg was recently reported from the Drau River, Austria (Hauer, 2003), and the first author viewed a prepared specimen (ca. 135 cm) caught from Plav Lake, Montenegro, in 2004 reported to weigh 37 kg. Huchen are extremely fast growing, putatively rarely reaching 20 years of age. Of 35 individuals ranging from 4.8 to 34.8 kg (85–144 cm) estimated ages ranged from 5 to 18 years (Hauer, 2003). Huchen are limited to submontane tributaries of the Danube (Holčik 1995), with sporadic occurrence in the main stem. Reports that Huchen naturally occur in other Black Sea tributaries such as the Dniester are not well substantiated as the species is not listed as naturally occurring there in Berg (1949) or Vasil'eva (2003), who reviewed the fauna of the region. While there is no hard data on the zoogeographic origins of Huchen, several authors have hypothesized that *H. hucho* is a geographic race of *H. taimen*, having colonized Europe post-glacially (Karaman, 1926; Berg, 1949). Berg (1949) assumed that *Hucho* once had a contiguous distribution from Asia to Europe, but went extinct in the Black Sea region, resulting in a vicariant distribution, and subsequent "speciation" of two lineages, now recognized as Huchen and taimen. Holčik (1990) estimated that Huchen have lost two-thirds of their distribution through the effects of river regulation, hydropower development and both industrial and agricultural pollution. Within Austria, Huchen occupy only 10% (ca. 450 river km) of their previous distribution, considering self-sustaining populations (Schmutz et al., 2002). As a top predator in large to mid-sized river systems, and a prized game fish, Huchen suffer from a variety of human-caused threats and are listed by the IUCN as endangered (Groombridge, 1994). In some parts of their range, Huchen are raised in small private fish farms and stocked to enhance fisheries, though there is no reliable data on the efficacy of these efforts (i.e. the relative contribution of stocked versus naturally reproduced individuals to populations), nor has there been any consideration given to their genetic diversity or potential geographic structure. Molecular data on *H. hucho* are scarce and mainly limited to phylogenetic analysis of a few individuals in higher order systematic studies (e.g. Crespi & Fulton, 2004; Phillips et al., 2004) or research focused on other related species (e.g. Froufe et al., 2003, 2005). Most recently, a conservation genetic study on geographically limited Huchen populations from Bavaria has been performed (Geist et al., 2009). However, no large-scale genetic analysis has been carried out on the species. Huchen habitat continues to be threatened by yet further hydropower development, and thus baseline genetic data are needed to support regional IUCN assessment or to guide future population specific conservation and research efforts. The authors, thus, present the first large-scale genetic characterization of Huchen, using a limited number of available samples from throughout most of their present range. Emphasis was placed on obtaining natural samples (that is from the wild, as opposed to hatchery material) from primarily adult individuals, and from as many different rivers systems as possible, in order to evaluate the potential for regional genetic structure.

Materials and methods

Sampling and DNA isolation

Forty-seven Huchen captured from 11 locations were non-invasively sampled from 2002 to 2007 (Table 1; Fig. 1). Samples stem from anglers or monitoring efforts in areas where Huchen are still known to reproduce. DNA was isolated from fin tissue preserved in 96% ethanol, using the Wizard Genomic DNA Purification Kit (Promega). Mitochondrial (mt) DNA sequence data from taimen, and both sharp- and blunt-snouted lenok *Brachymystax lenok* (Pallas, 1773), were used for outgroup comparison.

DNA amplification and sequencing

The PCR amplification of mtDNA control region (CR; ca. 1,300–1,700 bp) and NADH-1 (ca. 1,150 bp) was performed using primers and conditions reported in Razpet et al. (2007) and Froufe et al. (2005). Sequence reactions were prepared using a BigDye Terminator Ready Reaction Mix (PE Applied Biosystems) following manufacturer's recommendations. The whole CR was sequenced using the PCR primers, and in a separate reaction, an internal primer (STIF: CTTT TTTTTTTTTCCTTTCAGC) was used to allow double-strand sequencing of the difficult mono-"T" repeat block at position 552–565. New sequences were

Table 1 Summary of sampling localities for *H. hucho* including geographic coordinates, sample sizes for both mtDNA and microsatellite screening, and haplotype frequencies

Population/region	Geographic coordinates	Pop_code	Number of samples		Haplotype frequency									
			mtDNA	M_sats	Control region		NADH-1 gene				Combined haplotypes			
					Hh_CR1	Hh_CR2	Hh_ND1_1	Hh_ND1_2	Hh_ND1_3	Hh_ND1_4	Hap1	Hap2	Hap3	Hap4
Austria														
Drau River	46°43′N, 13°05′E	1	6	6	5	1	4	1	–	1	4	1		1
Gail River	46°36′N, 13°31′E	2	5	5	5	–	5	–	–	–	5			
Mur River	47°23′N, 15°18′E	3	4	4	4	–	3	–	1	–	3		1	
Slovenia														
Krka River	45°46′N, 15°00′E	8	5	5	5	–	–	–	5	–	–		5	
Sava River	46°05′N, 14°32′E	9	2	2	2	–	2	–	–	–	2			
Sora River	46°09′N, 14°21′E	10	2	2	2	–	2	–	–	–	2			
Bosnia-Herzogevenia														
Drina River	44°00′N, 19°15′E	4	7	7	7	–	7	–	–	–	7			
Vrbas River	44°20′N, 17°17′E	5	2	2	2	–	2	–	–	–	2			
Montenegro														
Lake Plav	42°35′N, 19°55′E	6	2	2	2	–	2	–	–	–	2			
Slovakia														
Vah River	49°21′N, 18°50′E	7	4	4	4	–	4	–	–	–	4			
Ukraine														
Tisa River	48°05′N, 22°57′E	11	3	8	3	–	3	–	–	–	3			
Total			42	47	42	1	35	1	6	1	34	1	1	6

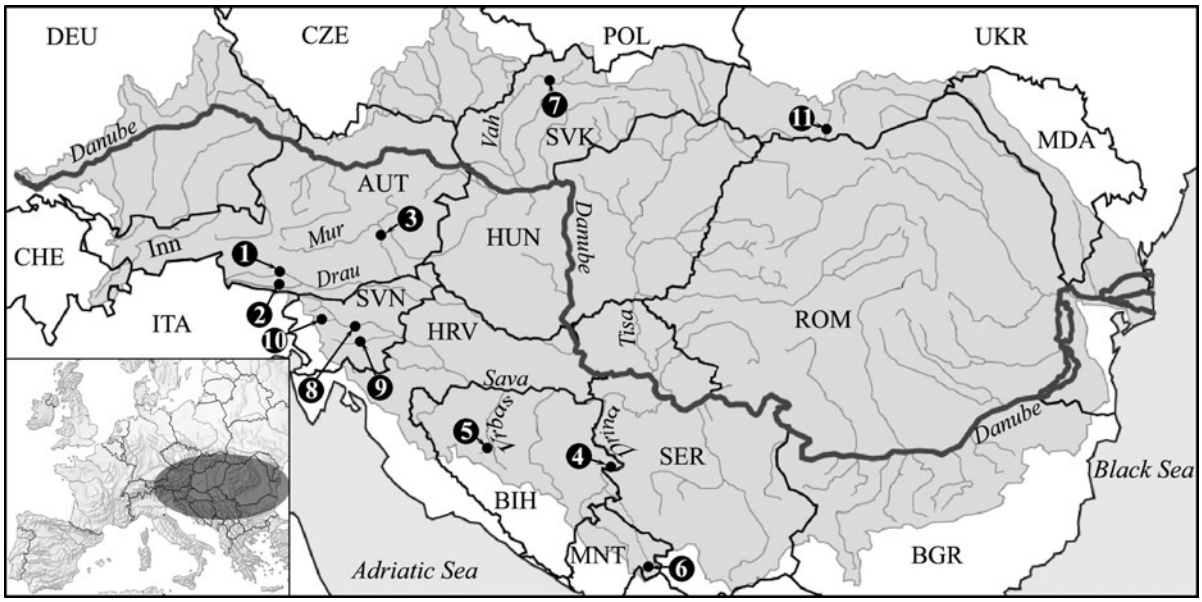

Fig. 1 Map of the Danubian basin, showing the original distribution range of *Hucho hucho* (in grey) as well as the sampling locations (numbered 1–11, and corresponding to the names listed in Table 1)

deposited in GenBank under accession numbers EU729360–EU729365 and EU760487–EU760491.

Sixteen microsatellite loci were tested (isolated from *Hucho*, *Brachymystax* and *Parahucho*; Froufe et al., 2004; Guangxiang et al., 2006) but only two (HLJZ003 and HLHZ023; Guangxiang et al., 2006) were polymorphic, non-duplicated, and revealed reliable electropherograms. These loci were screened in all 47 individuals using fluorescently labelled forward primers and conditions described in Jug et al. (2005). Annealing temperatures were 60°C for HLJZ003 and 55.5°C for HLHZ023.

Analysis

Sequences were aligned in Clustal W (Thompson et al., 1994) and adjusted by eye. Polymorphism was assessed using DNAsp ver. 4.10 (Rozas et al., 2003) and sequence divergence in MEGA version 2.1 (Kumar et al., 2001). Due to the low sample sizes, microsatellite variation was evaluated at the individual level analogous to a landscape genetic framework, with the aim of evaluating the potential relationship between individuals and geographic location. For this purpose, individual pair-wise genetic distances were calculated, with the proportion of shared allele distance (DAS distances, Bowcock et al., 1994). These distances were used both as input for construction of Neighbour-Joining (NJ) tree of individuals, using the program POPULATIONS (Langella, 2002), and in a correlation analysis with a matrix of pair-wise geographic distances. A geographic distance matrix based on great-circle distances between each site was derived and the correlation between these distances and genetic distances tested using a Mantel's test with statistical significance determined with 10,000 permutations. The choice of correlation coefficients was made after testing whether or not the matrices conformed to a normal distribution. These calculations were done using the software R-Package ver. 4 (Casgrain & Legendre, 2001).

As no population structure or assignment is assumed, statistical tests on regional structure were carried out post hoc, based on the structure revealed in the tree of individuals. A χ^2 test of independence was carried out on the distribution of mtDNA repeats with respect to this assumed geographic structure, and an allele permutation test (FSTAT, 10,000 permutations) was carried out on the microsatellite alleles for this same structure.

Results

The complete CR, 811 bp of the 5′-end of the ND1 gene, and 36 bp of flanking tRNA (*leu*) were aligned

Table 2 Frequency of the 82-bp repeats in the 3′-end of the mtDNA control region across 11 sample sites of *H. hucho*

Population/region	Number of 82-bp long repeats in the control region						
	5	6	7	8	9	10	Total
Austria							
Drau River	–	2	1	3	–	–	6
Gail River	–	2	2	1	–	–	5
Mur River	1	–	1	1	–	1	4
Slovenia							
Krka River	–	–	–	5	–	–	5
Sava River	2	–	–	–	–	–	2
Sora River	–	–	2	–	–	–	2
Bosnia-Herzogevenia							
Drina River	–	–	–	6	1	–	7
Vrbas River	–	–	–	–	2	–	2
Montenegro							
Lake Plav	–	–	–	2	–	–	2
Slovakia							
Vah River	–	–	–	3	1	–	4
Ukraine							
Tisa River	–	–	–	3	–	–	3
Total	3	4	6	24	4	1	41

in 41 individuals of *H. hucho*, one *H. taimen* and two *B. lenok* (sharp- and blunt snouted). All individuals contained an 82-bp repeat in the 3′-end of the CR analogous to that reported in coregonids (Brzuzan, 2000), and other salmonids including *Thymallus*, *Brachymystax*, *Hucho* and *Salmo* (Weiss et al., 2002; Sušnik et al., 2006, 2007). In *H. hucho*, this repeat occurred between 5 and 10 times (Table 2), while it occurred 3 times in *H. taimen* and sharp-snouted *B. lenok* and 4 times in blunt-snouted *B. lenok*. Eight repeats were the most common motif ($N = 24$), occurring across all regions. Austria and Slovenia revealed more diversity, with all six repeat variants found. All samples from outside Austria and Slovenia exhibited either eight or nine repeats. As the mutation mode of this repeat is unknown and most probably involves intra-molecular mechanisms (Sušnik et al., 2007) all but one copy were removed from further phylogenetic analysis. The remaining CR sequence revealed 60 variable sites and eight, 1-bp long indels. There were 11 fixed differences (1.1%) between *H. taimen* and *H. hucho*, 18 fixed differences between *Hucho* and *Brachymystax* (1.8%) and a single polymorphic position within *H. hucho* resulting in only two CR sequence haplotypes in the species (GenBank acc. numbers EU729360, EU729361). For the ND1 gene, there were 91 variable sites, with 15 fixed differences between *H. taimen* and *H. hucho* (1.8%), 32 fixed differences between *Hucho* and *Brachymystax* (3.7%) and four polymorphic positions within *H. hucho*, leading to four haplotypes (GenBank acc. numbers EU729362–EU729366), each separated by one to two substitutions. Including outgroups, there were only four non-synonymous changes, three of which were within the genus and two within *H. hucho*. Combining the two sequences, there were a total of four haplotypes (Table 1), the same number found for the ND1 gene alone. One haplotype was dominant and found in all regions (Hap1), whereas two haplotypes (Hap 2 & 4) appeared in single individuals from the Drau River, and Hap 3 was fixed in the Krka River, but also found in one individual from the Mur River. Surprisingly, three of the four haplotypes occurred among the six individuals from the Drau River, Austria (Table 1).

The two microsatellite loci revealed 6 and 11 alleles per locus, respectively. The most frequent allele (41%) at locus HLJZ003 was not found in any sample from Austria or Slovenia. Large positive F_{is}-values for both loci (0.507, 0.452, $P < 0.001$), reflecting extreme heterozygote deficiency globally ($H_E = 0.721$ and 0.839 vs. $H_O = 0.361$ and 0.468,

Table 3 Allele frequencies and expected (H_E) and observed (H_O) heterozygosities for the two microsatellite loci screened in 47 individuals of *H. Hucho*, from 11 locations throughout the Danubian drainage

Locus	Allele size	Frequency	H_E	H_O	F_{is}	P
HLJZ003			0.721	0.361	0.507	<0.001
	258	0.223				
	264	0.415				
	267	0.010				
	270	0.213				
	273	0.043				
	279	0.096				
HLHZ023			0.839	0.468	0.452	<0.001
	308	0.021				
	312	0.287				
	314	0.117				
	322	0.032				
	326	0.128				
	338	0.159				
	342	0.117				
	346	0.064				
	356	0.011				

respectively), strongly support that multiple populations were sampled (Table 3). This inference was further supported in that there was no allele-size specific pattern to the heterozygote deficiency, but rather a strong geographic component supporting the existence of distinct populations, i.e. samples from Austria and Slovenia form a group distinguishable from all remaining samples (Fig. 2). There was no further clear grouping of individuals within these two groups, and due to limited sample sizes, no attempt was made to estimate any population genetic parameter within or between these sample sites, or even to suggest how many populations have been sampled.

Both genetic and geographic distances were non-normally distributed (Kolmogorov–Smirnov test, $P < 0.01$) and thus non-parametric correlation coefficients were used for a Mantel's test. Pair-wise distances between sites ranged from 35 to 757 km. The correlation between genetic and geographic distances was moderately low, but nonetheless highly significant (Spearman's ρ 0.428, $P < 0.0001$; Kendall's τ 0.339, $P < 0.0001$). Based on the structure shown in Fig. 2, an allele permutation test between these two groups of sampling sites was highly significant ($P < 0.0001$)

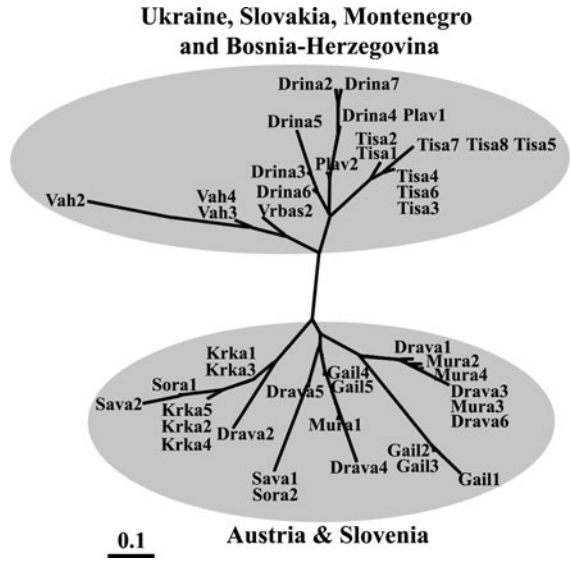

Fig. 2 Tree of individuals based on allele sharing distances (DAS) across two microsatellite loci

and the distribution of mtDNA repeats between these same groups was also significant ($\chi^2 = 18.2$; df = 5; $P = 0.003$).

Discussion

The Danubian basin is known as perhaps the most important refuge for freshwater organisms in Europe and cold-tolerant species with wider European distributions often reveal their highest levels of mtDNA haplotype diversity and/or presumed oldest lineages in the region [e.g. *Thymallus thymallus* (Linnaeus, 1758), Weiss et al., 2002; *Perca fluviatilis* (Linnaeus, 1758), Nesbø et al., 1998; *Salmo trutta* (Linnaeus, 1758), Bernatchez & Danzmann, 1993]. Contrary to this notion, extremely low and geographically uniform levels of mtDNA haplotype diversity was revealed in Huchen throughout their distribution, as only four closely related haplotypes were identified from screening over 1,800 bases (excl. repeats).

There are at least four not necessarily mutually exclusive hypotheses explaining the limited mtDNA variation found in Huchen: (1) a slow molecular clock; (2) low historical effective population sizes; (3) a speciation founder effect; (4) more recent human-caused bottlenecks. We argue that the molecular clock is playing a role in the current observation

of limited genetic diversity in the species, while additional causes cannot be eliminated. Froufe et al. (2003, 2005) postulated a slower molecular clock in *Hucho*, based on comparative phylogeographic data across three genera. The correlated characteristics of body size, metabolic rate and temperature would also support a slower mutation rate in the large-bodied *Hucho* (Martin & Palumbi, 1993; Gillooly et al., 2005). Additionally, strong evolutionary constraints on the salmonid CR have been reported (Froufe et al., 2003, 2005) and there is likely purifying selection on the NADH-1 gene in the genus, evidenced by the extremely low number of non-synonymous substitutions reported for taimen (Froufe et al., 2005). More recent bottlenecks as well as historically low effective population sizes are unlikely to result in the observation of four very closely related haplotypes across the species range. A more ancient and very strong bottleneck resulting in a single ancestral haplotype followed by more recent mutation cannot be ruled out. A species founder effect could only be argued for in addition to a very slow molecular clock. The two sister species of *Hucho* display almost 2% divergence in mtDNA, and thus, even at the 1%/MY divergence rate estimated for the most rapidly evolving of the three genera (*Thymallus*; Froufe et al., 2003), 2 million years separation should be ample time for production of mtDNA haplotype diversity. We cannot exclude that stocking and transport has had some peripheral effects on the data, but across the whole range of the species in six countries this is highly unlikely.

Despite these low level of molecular diversity for the species, the results of this study also support significant geographic structure associated with multiple populations among major drainages; there appears to be a general division between northern (Austria and Slovenia) samples compared to those further downstream in the Danube catchment (Bosnia-Herzegovina and Montenegro), based on high frequency private msat alleles as well as mtDNA repeats.

The regional genetic structure implied by our data should be respected by managers, who may be tempted to transport brood fish or yearlings across the range of the species distribution. While the genetic differentiation of individuals captured within present governmental boundaries appears low to non-existent with the set of genetic markers applied, we still advocate a conservative approach in rearing and stocking the species, to limit cross-basin transport to an absolute minimum. Current conservation efforts are focused on several river systems in Austria and Slovenia, such as the Mur and Drau rivers where hydropower developers challenge, at least in some river reaches, the native status of Huchen claiming its presence stems only from stocking. In addition to further sampling, from both private breeders and from the wild, a high-resolution screening protocol based on reliable species-specific microsatellite loci as well as mtDNA repeats is under development and will be needed to evaluate the source of wild-caught Huchen in these regions where its origins are in dispute.

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