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The impact of stocking on the genetic structure of European grayling (*Thymallus thymallus*, Salmonidae) in two alpine rivers

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Abstract

The European grayling (*Thymallus thymallus*) is considered to be threatened in several European regions. In recent decades fishery managers have increasingly turned to stocking programs as one way to combat the negative effect that human influence has had on population densities. The present study surveyed the genetic structure of two Danube drainage populations at the Inn and Drau rivers, in Austria, on the basis of mtDNA sequences. Data were placed in the context of the phylogeographic structure of European *Thymallus* and thus could reveal unexpected geographical mixing due to stocking with allochthonous individuals. Our analyses revealed that regular stocking of fish not originating from their natal Rivers has left genetic traces in both systems surveyed. These traces may be classified as marginal for the Inn river and its tributaries in which 97% of the graylings investigated carried haplotypes belonging to the northern alpine lineage, corresponding to the region through which the Inn flows. In contrast, the genetic composition of the Drau population, situated in the southern Alps, has been seriously altered through the stocking of fish belonging to the northern alpine mtDNA lineage as only 62% of the fishes sampled carried haplotypes representing the native southern alpine lineage.

Introduction

A series of studies on the ecology, biology, and molecular genetics of European grayling (*Thymallus thymallus*) populations point to the acute endangerment of this species in several water bodies (Uiblein et al., 2000, 2001; Koskinen et al., 2001, 2002a, b). The causes are pollution, the construction of hydroelectric facilities that both inhibit migration and dispersal as well as alter flow regimes, river bed channelization, overfishing, and in some specific localities bird predation or competition from introduced rainbow trout, *Oncorhynchus mykiss* (Weiss et al., 2002). Human influence has so greatly affected grayling populations that fishery managers have recently turned to intensive stocking efforts in an attempt to restore and maintain stocks. Numerous molecular based studies have demonstrated a clear phylogeographic structure for European salmonids, primarily reflecting current drainage systems but also the past effects of Pleistocene glacial cycles (Bernatchez et al., 1992; Bernatchez 2001; Koskinen et al., 2002a; Duftner et al., 2003). For *Thymallus*, however, this structure is exceedingly complex, particularly in the upper Danube where multiple divergent lineages are found, most likely reflecting isolated glacial refugia (Weiss et al., 2002). The occurrence of such a pattern is assumed to be a consequence of grayling's limited dispersal ability and relatively narrow ecological niche. This is further supported by the fine-scaled population substructuring of grayling reported in Scandinavian populations (Koskinen et al., 2001, 2002b). Thus, stocking with individuals from non-autochthonous populations might have severe effects on the genetic structure of local populations in *Thymallus* and seriously threaten their maintenance through the deleterious effects of inbreeding and outbreeding resulting in reduced fitness to local conditions (Avise & Hamrick, 1996).

The phylogeographic structure of European grayling was recently elucidated in a study of Weiss et al. (2002) including 44 populations throughout the western-European range of the species. The authors demonstrated the presence of multiple divergent mtDNA clades in European rivers, most of which were assigned to particular sections of drainage systems. This finding supports the notion that European grayling have had a long history in Western Europe, pre-dating Pleistocene glacial cycles. Their intraspecific phylogenetic tree revealed two well-supported clades within the Danube basin, one highly divergent clade in the Adriatic basin, and one large, diverse group representing most other central- and western-European populations. A deeply divergent haplotype fixed in the Loire basin in Central France, more groups of haplotypes from distinct Danubian tributaries, and a relatively ancestral haplotype fixed in former tributaries of the Elbe in Denmark all suggest a complex pattern of inter- and post-glacial expansions originating from disjunct refugia throughout Central Europe. Despite some evidence for human-mediated stock transfers, the authors suggest by means of nested clade analysis (Templeton, 1998) that corridors across drainage systems such as the lower Rhine (Moselle) and Elbe served as sources for expansion into the Baltic to the north, as well as the upper Rhine and Danube to the south. Moreover, specific Rhine populations (Doller, Orbe, and Reuss) served as sources of colonization of the Rhone. Concerning the distribution of mtDNA lineages in the Danube system, Weiss et al. (2002) found that the Danube and its tributaries north of the Central Alps comprise two mtDNA lineages, and that the Danube and its tributary south of the Central Alps is inhabited by a third distinct mtDNA lineage, as well as the Danube in Slovenia, which again comprises another mtDNA lineage. In consequence, it was argued that the current trends to trans-locate brood stocks for rearing and release in response to the decline of natural populations must be reconsidered in order to protect the natural diversity of this species.

Here we report the results of a mtDNA based study on the haplotype distribution of European grayling in two Austrian river systems, the Inn river, belonging to the northern alpine Danube drainage, and the Drau River, belonging to the southern alpine Danube drainage. Both rivers are known to receive regular inputs of hatchery grayling, the origin of which is traceable for the majority of stocks, at least for the most recent past (5–10 years). The records for the Inn River covering our sample sites document the release of ca. 100 000 yearling grayling per year within the last 6 years, all originating from a single hatchery that uses only brood stock captured from the Inn itself. In the Drau River system several thousand grayling of Inn origin were stocked 20 years ago, and during the last 5 years the water body has been managed through the release of 25 000 yearlings per year raised from brood stock of Drau origin. However, we note that both river systems are quite large and contain many independently managed reaches in which fish of unknown origin might have been released.

We aimed to evaluate grayling from the Inn and Drau river systems in the context of the mtDNA phylogeographic structure of European *Thymallus* and thereby assess the extent of influence that stocking has had on the occurrence of expected mtDNA lineages.

Materials and methods

In total, 94 individuals were collected from nine localities in two Austrian Danube river tributaries – Inn and Drau – north and south of the Central Alps. Detailed sample information is given in Table 1 (for a map of sampling localities see Fig. 1). For all 94 specimens we sequenced the second section of the mitochondrial control region (457 bp, base positions 631–1087 of the complete control region), and for 51 individuals we se-

Table 1. Characterization of samples including river, sampled locality, major river drainage, haplotype, number of individuals, and GenBank accession number

River	Locality	System[a]	Haplotype[b]	R[d]	N[e]	GenBank Accession No.
Isel	Ainet	Drau	Da1	NA	1	AF522395[f]
Isel	Ainet	Drau	Da4	NA	1	AF522398[f]
Große Drau	Lienz	Drau	Da1	NA	2	AF522395[f]
Große Drau	Lienz	Drau	Da5	SA	5	AF522399[f]
Große Drau	Lienz	Drau	Da7	SA	2	AF522401[f]
Große Drau	Lienz	Drau	Da11	NA	1	AF522405[f]
Große Drau	Lienz	Drau	Da15	SA	1	AF522409[f]
Große Drau	Lienz	Drau	-[c]	SA	1	AY594183
Große Drau	Nikolsdorf	Drau	Da1	NA	2	AF522395[f]
Große Drau	Nikolsdorf	Drau	Da4	NA	1	AF522398[f]
Große Drau	Nikolsdorf	Drau	Da5	SA	6	AF522399[f]
Große Drau	Nikolsdorf	Drau	Da7	SA	4	AF522401[f]
Große Drau	Nikolsdorf	Drau	Da11	NA	2	AF522405[f]
Kohlenbach	Kössen	Inn	Da1	NA	6	AF522395[f]
Kohlenbach	Kössen	Inn	Da11	NA	6	AF522405[f]
Kohlenbach	Kössen	Inn	new	M	1	AY594181
Inn	Pfunds	Inn	Da1	NA	4	AF522395[f]
Inn	Pfunds	Inn	Da11	NA	1	AF522405[f]
Inn	Pfunds	Inn	-[c]	NA	5	AY594184
Inn	Haiming	Inn	-[c]	NA	8	AY594185
Inn	Zirl	Inn	Da1	NA	2	AF522395[f]
Inn	Hall	Inn	Da7	SA	2	AF522401[f]
Inn	Hall	Inn	new	NA	1	AY594182
Inn	Hall	Inn	-[c]	NA	16	AY594186
Inn	Hall	Inn	-[c]	M	1	AY594187
Inn	Kirchbichl	Inn	-[c]	NA	12	AY594188

[a] River system to which river sampled belongs to. According to Weiss et al. (2002) the Inn-system is assigned to the Danube drainage (Northern Alps) and the Drau-system is assigned to the Danube drainage (Southern Alps).

[b] Haplotypes of complete mitochondrial control region sequences as defined by Weiss et al. (2002).

[c] Only the second part of the mitochondrial control region was available.

[d] mtDNA lineage to which the samples were assigned to based on the complete or the second part of the mitochondrial control region: NA, Danube drainage (Northern Alps); SA, Danube drainage (Southern Alps); M, Mixed Rhine/Danube drainage.

[e] Number of individuals per haplotype and sampling locality.

[f] GenBank accession number according to Weiss et al. (2002).

quenced the entire mitochondrial control region (1087 bp).

DNA-extraction, polymerase chain reaction (PCR), and automatic sequencing followed standard protocols (Bruford et al., 1998; Weiss et al., 2001). The primers for PCR-amplification and chain termination sequencing for the first section of the mitochondrial control region were LRBT-25, 5′-AGA GCG CCG GTC TTG TAA TC-3′ (Uiblein et al., 2001) and the newly designed primer INT-R, 5′-CGA CCT TAT TAG TTC TTC TTA G-3′. For the second section of the mitochondrial control region we used the primers INT-F, 5′-AGC CGG GCG TTC TCT CCT ATA T-3′, and LRBT-1195, 5′-GCT AGC GGG ACT TTC TAG GGT C-3′ (Uiblein et al., 2001). DNA fragments were visualized on an ABI 3100 automatic sequencing system. DNA sequence alignment was performed using the program CLUSTAL W (Thompson et al., 1994). The 51 DNA sequences of the whole mitochondrial control region were added to the data set of Weiss et al. (2002) to assign them to

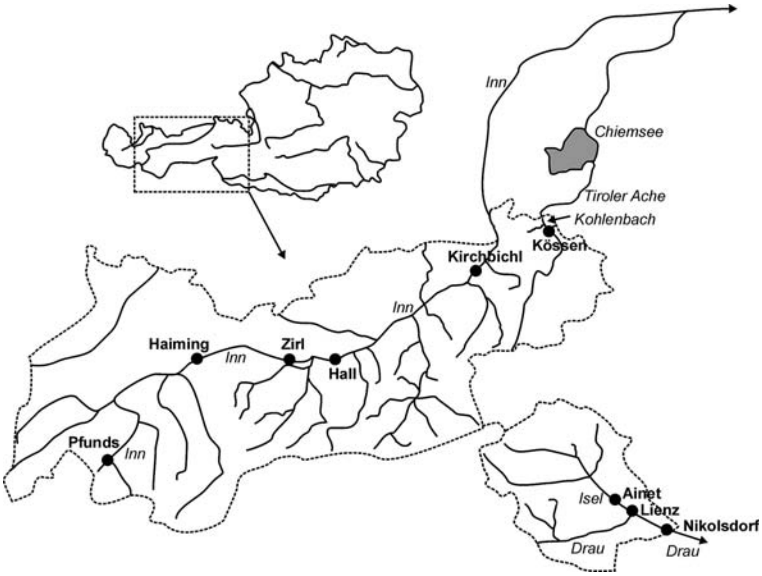

Figure 1. Map of the Inn- and Drau-river system in the Tyrol, Austria, showing the sampling localities.

previously identified European grayling lineages. Sequences of all haplotypes, described herein, are available from GenBank (see Table 1).

First, a phylogenetic tree was constructed based upon the entire control region by applying the neighbor joining algorithm (NJ) implemented in PAUP*4.0b3 (Swofford, 2000) (Fig. 2). For NJ, the substitution model $HKY + I + \Gamma$ (Hasegawa et al., 1985; base frequencies A, 0.3203; C, 0.2127; G, 0.1475; T, 0.3195; gamma shape parameter α, 0.9043; proportion of invariable sites I, 0.7761; transition/transversion ratio, 2.6238) was used, since it was chosen as the most appropriate model of sequence evolution by Modeltest (Hierarchical Likelihood Ratio Tests; Posada & Crandall, 1998). *Thymallus arcticus*, *T. brevirostris*, and *T. grubii* were declared as outgroup taxa. The base positions 603–639 were excluded from the analysis due to ambiguities in the alignment. As a measure of confidence we performed bootstrapping (Felsenstein, 1985) with 1000 pseudo-replicates. In a second step of analysis a NJ analysis based upon the second section of the control region (substitution model HKY; base frequencies: A, 0.3351, C, 0.1944, G, 0.1527, T, 0.3178; transition/transversion ratio, 1.4625) was conducted for 94 specimens, again declaring *Thymallus arcticus*, *T. brevirostris*, and *T. grubii* as outgroup taxa.

For this data set, a minimum spanning network was constructed in order to visualize the number of base substitutions separating the mtDNA haplotypes and lineages (Fig. 3). The network is based on one most parsimonious tree [un-weighted maximum parsimony analysis; tree length, 66 base substitutions; consistency index excluding uninformative characters, 0.73; retention index, 0.83; rescaled consistency index, 0.69] that was most similar to the NJ tree.

Results

The NJ tree, based on the data set of Weiss et al. (2002) plus 51 new sequences of the complete mitochondrial control region from the Drau- and Inn-system, shows that all individuals sequenced can be assigned to the three previously identified mtDNA haplotype groups present in Austrian rivers: the northern alpine Danube drainage clade, the southern alpine Danube drainage clade, and the mixed clade from the Rhine and Danube (Fig. 2). In total, nine haplotypes were found in the newly sequenced samples from the Inn- and the Drau-system. Most individuals could be assigned to previously identified haplotypes (Dal, Da4, Da5, Da7, Dal1, Dal5; Weiss et al., 2002), but two individuals, one from the Kohlenbach River at

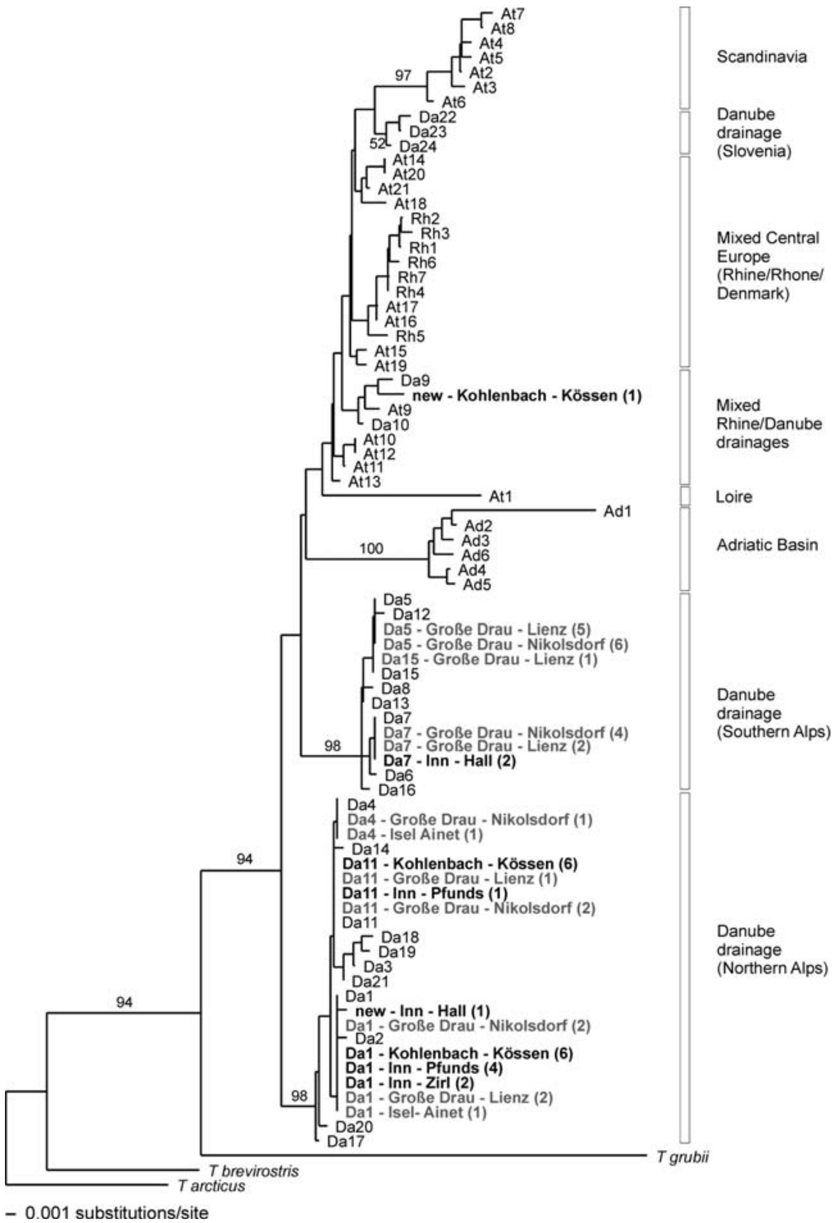

Figure 2. NJ phylogram based on the data set of Weiss et al. (2002) including 58 haplotypes plus 52 new sequences of the complete mitochondrial control region from the Drau- and Inn-system using the $HKY + I + \Gamma$ model of molecular evolution, to assign the haplotypes found in the Inn-and Drau River to major clades defined by Weiss et al. (2002). Two individuals, one from the Kohlenbach at Kössen and one from the Inn River at Hall, represented new haplotypes, denoted by 'new'. Node support for the major clades defined by Weiss et al. (2002) is shown in the form of bootstrap values (1000 replicates) for NJ at the branches. Bold taxa represent newly sampled haplotypes (grey, Drau-system; black, Inn-system).

Kössen and one from the Inn River at Hall, represented new haplotypes. While the new haplotype from Inn clustered within the clade comprising haplotypes from the northern alpine Danube drainage system, the new haplotype from Kohlenbach was assigned to the mixed group containing haplotypes from the Danube- and Rhine drainage system.

Haplotypes could be unambiguously assigned to the three distinct clades regardless of whether

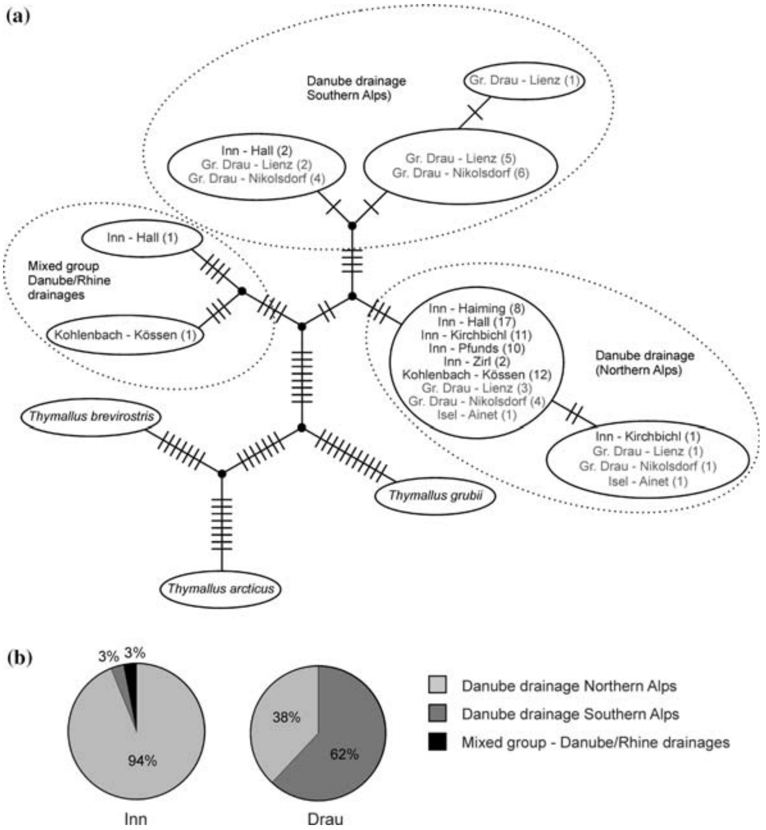

Figure 3. (A) Minimum spanning tree based on 94 DNA sequences (plus three outgroup taxa: *Thymallus arcticus*, *T. brevirostris*, and *T. grubii*) of the second section of the mitochondrial control region (457 bp, base positions 631–1087 of the complete control region). Bars indicate the number of base substitutions among haplotypes assigned to three mtDNA lineages – Danube lineage north of the central Alps, Danube lineage south of the central Alps, and Mixed lineage Danube and Rhine (grey, Drau-system; black, Inn-system). Origin and frequency (in parentheses) of haplotypes are given. (B) Pie diagrams depicting frequencies of mtDNA lineages for the Inn- and Drau river.

the complete or partial control region sequence was used. Thus, for summary purposes we consider the partial sequence in order to encompass the total sample (94 individuals). From the 65 individuals sampled in the Inn drainage, 61 (94%) were assigned to the northern alpine clade, 2 (3%; from Kohlenbach at Kössen and Inn at Hall) were assigned to the mixed clade of the Rhine- and Danube system, and two individuals (3%; Inn at Hall) were assigned to the southern alpine clade. Out of the 29 individuals collected from the Drau system, 18 specimens (62%) were assigned to the southern alpine clade, whereas the remaining 11 individuals (38%) were attributed to the northern alpine haplotype group.

The minimum spanning tree (Fig. 3), based on 94 sequences of the partial control region shows the number of substitutions between haplotypes and highlights the high genetic divergence among the three mtDNA lineages: A minimum of 12 base substitutions separate haplotypes of the northern alpine Danube drainage clade from the mixed clade of the Rhine-and Danube drainage, a minimum of 14 substitutions were counted between the southern alpine Danube drainage haplotype group and the mixed clade of the Rhine- and Danube drainage, and at least eight substitutions between the two Danube drainage lineages north and south of the Alps.

Discussion

The population decline of European grayling throughout central Europe is considered to be in a crisis phase, which has led to an increase in management activities involving the rearing and stocking of fish, with little input from biologists in terms of genetic or ecological considerations. A conservation-oriented management framework must consider the genetic architecture of the natural populations, the source of the stocking material, and a proper breeding design that maintains genetic variation. To achieve this framework, basic genetic data are needed on the current status of both natural and hatchery stocks. This study provides an overview of the existing lineages of grayling in two major Austrian river systems subject to intense management activities. The introduction of allochthonous material in the Drau system has clearly left a genetic imprint in that 38% of the individuals screened carried a mtDNA control region haplotype belonging to the northern alpine clade. The mean sequence divergence between the northern and southern alpine clades has been shown to be 1.4% (Weiss et al., 2002). Based on a substitution rate calibration specific for *Thymallus* (Koskinen et al., 2002), this divergence corresponds to at least one million years. This is a considerable time period for both local adaptations and (historical) chance events to occur and thus we must assume that these two lineages are evolutionarily distinct and should be managed as separate units (Moritz, 1999). Thus, we conclude that the upper Drau system has been seriously affected by the stocking of a highly divergent albeit Danubian lineage, in agreement with the stocking record documenting a massive introduction event about 20 years ago, albeit local mother fish are used since 5 years. The long-term presence of mtDNA haplotypes from an allochthonous lineage in such percentages strongly implies that introgression has taken place and the original genetic architecture of the native Drau river stocks has been compromised.

Even if a similar level of stocking has been carried out in the Inn drainage, it is not reflected in the frequency distribution of mtDNA haplotypes. At least 94% of the individuals screened can be assigned to the northern alpine mtDNA clade, whereby two additional individuals (then 97%) belong to another clade that may in fact represent an autochthonous lineage in the region. This so-called 'mixed Rhine-Danube' clade was first defined based on a small sample of individuals found in the Saalach and Vöckla rivers in Austria, various samples from the Rhine catchment in Switzerland, as well as former tributaries of the Elbe system in Denmark (Weiss et al., 2002). Following the authors' biogeographic interpretation, this clade represents a distinct central European refuge from which fish have expanded post-glacially into distinct regions of the upper Danube system. The presence of two individuals (3%) from the southern alpine clade in the Inn drainage reflects a minimal influence of stocked allochthonous lineages. While the complete stocking history in large Austrian systems is nearly impossible to track, it appears from our results that the Inn system has received stocked fish primarily from broodstocks stemming from the northern alpine mtDNA lineages, in agreement with the stocking record. However, it is also possible that fish stocked into the Inn have simply not survived or interbred with native stocks and thus introgression has not occurred (hybrids among lineages cannot be detected with mtDNA due to its maternal pathway of inheritance). Moreover, it should be mentioned that mtDNA data reflect a regional phylogeographic perspective, but do not allow to address more fine-scale genetic relationships within and among local populations.

It is noteworthy to mention that despite the historical records of extensive stocking activities, no haplotypes of geographically distant origins have been detected. This is in contrast to the documentation of distant lineages appearing in rivers of France, for example (Weiss et al., 2002), and our knowledge of the use of imported material in some Austrian hatcheries. Thus, it appears that Danube drainage lineages have been almost exclusively used in Austrian management activities.

As sustainable conservation-oriented strategy for Austrian grayling populations will require both additional population-level genetic data as well as assessment of the ecological conditions and degree of natural reproduction. Aquatic habitats must be adequate to support the full life-history cycle of the organism for any management strategy beyond a 'put-and-take' fishery to make sense. From the genetic perspective, it is not only necessary to use stocking material of local origin, but also the phylogeographic integrity and the level of genetic variation in brood stocks must be monitored, or more ideally, the use of 'broodstocks' completely avoided. As an alternative, gametes can be collected in the wild and yearling fish raised and released into the habitats from which the gametes came.

While the screening of mtDNA lineages provides a valuable overview of the mixture of geographically distinct lineages, an expanded role for genetics in the future management of Austrian salmonids must be based on multi-locus data (e.g. microsatellites; Weiss et al., 2002). Since cross-breeding (or natural reproduction) cannot be directly assessed on the basis of mtDNA, only nuclear DNA markers allow to directly estimate the level of introgression among distinct lineages. Such data are also needed to assess the fine-scale level of genetic variation, the degree of genetic substructure within large systems such as the Drau and Inn, or genetic loss within hatchery stocks. These data can also be used for choosing brood fish. This approach is presently being pursued on the Soca River, Slovenia, where a similar problem has been created in that Danube drainage (Sava) strains have been stocked into the Soca (Adriatic drainage) where a highly distinct lineage of grayling exists (Sušnik et al., 2001). Here, the future management strategy is to selectively rear fish exhibiting the least degree of introgression from allochthonous lineages.

While genetic considerations are playing an increasingly important role in conservation-oriented management schemes it can not be understated that a broad range of primarily ecological factors are the primary cause of the decline of salmonid populations in Austria (Uiblein et al., 2000; Weiss et al., 2001) and until these causes are addressed, the sustainability of any management plan must be held in question.

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