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Sixty years of anthropogenic pressure: a spatio-temporal genetic analysis of brown trout populations subject to stocking and population declines

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Abstract

Analyses of historical samples can provide invaluable information on changes to the genetic composition of natural populations resulting from human activities. Here, we analyse 21 microsatellite loci in historical (archived scales from 1927 to 1956) and contemporary samples of brown trout (*Salmo trutta*) from six neighbouring rivers in Denmark, to compare the genetic structure of wild populations before and after population declines and stocking with non-local strains of hatchery trout. We show that all populations have been strongly affected by stocking, with admixture proportions ranging from 14 to 64%. Historical population genetic structure was characterized by isolation by distance and by positive correlations between historical effective population sizes and habitat area within river systems. Contemporary population genetic structure still showed isolation by distance, but also reflected differences among populations in hatchery trout admixture proportions. Despite significant changes to the genetic composition within populations over time, dispersal rates among populations were roughly similar before and after stocking. We also assessed whether population declines or introgression by hatchery strain trout should be the most significant conservation concern in this system. Based on theoretical considerations, we argue that population declines have had limited negative effects for the persistence of adaptive variation, but admixture with hatchery trout may have resulted in reduced local adaptation. Collectively, our study demonstrates the usefulness of analysing historical samples for identifying the most important consequences of human activities on the genetic structure of wild populations.

Keywords: admixture, effective population size, gene flow, local adaptation, microsatellite DNA, stocking

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Introduction

Analysing population genetic structure based on contemporary samples provides mainly a short-term perspective of evolutionary and demographic processes. Although coalescence-based methods now permit 'looking back in time' based on samples taken at a single time-point (Storz & Beaumont 2002), such methods nevertheless make specific assumptions regarding population stability and demographic processes. Moreover, human activities such as habitat degradation, overexploitation and population translocations may have severely affected the genetic composition and structure of natural populations over time (Bouzat *et al*. 1998; Dawson *et al*. 2005; Smith *et al*. 2008). Hence, without temporal samples, it cannot be assumed that current genetic structure reflects past genetic structure. In order to understand natural evolutionary processes and to assess anthropogenic impact, it is therefore of interest to analyse temporal samples and estimate demographic and population genetic parameters over time (Schwartz *et al*. 2007).

Analysis of DNA from historical samples, such as archived skin, teeth, feathers or fish scales represents a powerful approach for expanding the temporal scale of population studies (Wandeler *et al*. 2007; Leonard 2008; Nielsen & Hansen 2008). These sources of DNA typically encompass timescales ranging from decades to centuries

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and are increasingly being used in population genetic studies of mammals (Pichler & Baker 2000; Pertoldi *et al*. 2001), birds (Bouzat *et al*. 1998; Martinez-Cruz *et al*. 2007) and fishes (Nielsen *et al*. 1997; Hansen *et al*. 2002; Guinand *et al*. 2003; Fraser *et al*. 2007a).

Salmonid fishes (e.g. salmon, trout) are subdivided into discrete populations, which are often connected by some degree of gene flow. Both theoretical considerations and empirical results from a number of species show that salmonid populations may be adapted to local environments (Taylor 1991). Their demands for unpolluted water and access to suitable spawning sites render them vulnerable to habitat degradation. Moreover, they are of high economical and recreational value, which has led to stocking of wild populations with hatchery-reared conspecifics in order to compensate for population declines. However, salmonid hatchery strains have often been subject to intentional or unintentional domestication selection, such that their fitness in the wild may decrease considerably with each generation of hatchery-rearing (Araki *et al*. 2008). Stocking with nonlocal strains is considered especially problematic because in addition to the potential for a loss of wild fitness during hatchery-rearing, these may not possess locally adapted characteristics associated with the environments into which they are stocked. Results from empirical studies of the genetic impact of local and nonlocal hatchery salmonids have generally supported that they exhibit reduced fitness in nature relative to wild fish (Mclean *et al*. 2003; Ruzzante *et al*. 2004; Caroffino *et al*. 2008). Nevertheless, the outcomes of stocking in terms of hatchery–wild introgression range from near zero introgression to almost complete displacement of native populations, even with stocking of the same hatchery strain (Hansen 2002).

Most studies of genetic interactions between wild and hatchery fish have focused on the impact within individual wild populations (Hansen 2002; Mclean *et al*. 2003). Stocking may also significantly affect wild population structure by decreasing genetic differentiation (Eldridge & Naish 2007), but it is not known whether this is due entirely to direct introgression by hatchery fish, or due at least in part to increased rates of gene flow following admixture. For instance, hatchery fish often exhibit weaker homing than wild fish (Jonsson & Jonsson 2006), and in some cases significant numbers of stocked hatchery fish have been recovered in other rivers than those into which they were stocked (Vasemägi *et al*. 2005). It is not known whether these higher straying rates reflect circumstances of stocking or genetically based differences among populations, but large-scale reciprocal transplantation experiments among different brown trout (*Salmo trutta*) populations have indeed suggested heritable differences in migratory patterns and straying rates (Svärdson & Faderström 1982). The resultant concern is that stocked hatchery fish may act as a 'bridge' for disrupting reproductive isolation between wild populations (Ferguson & Taggart 1991). Hence, information is needed on whether and how hatchery fish might affect: (i) wild population genetic structure per se; (ii) interpopulation gene flow; and (iii) adaptation at local and regional scales (i.e. within-rivers vs. over multiple-rivers).

Our study assesses these three potential hatchery–wild interactions using exemplar data on anadromous brown trout (*S. trutta*) populations from western Jutland, Denmark (see Fig. 1). The rivers in this region encompass the largest (Skjern River) and fourth largest (Storaa) drainage in Denmark and were previously inhabited by sizable populations of brown trout and Atlantic salmon (*Salmo salar*). Historical catch data from the Sneum River indicate that up to 3000 kg of sea trout were harvested annually from 1945 to 1954 (Larsen 1993), suggesting an annual spawning run in the thousands (assumed mean weight per individual = 3 kg). Since the 1950s, over-fishing and habitat degradation have caused population declines. As an example, capture–mark–recapture estimates from the Sneum River in 1995 showed that the spawning run had declined to 390–766 individuals (Dieperink *et al*. 1997). Subsequently, the rivers were stocked with hundreds of thousands of hatchery trout from primarily two strains until this activity was substituted by supportive breeding of local populations in the late 1990s. Given the combined effects of population declines and stocking activity, it has been questioned whether any remnants of indigenous populations persist. Fortunately, archived scale samples from 1927 to 1956 were available from anadromous brown trout from six neighbouring rivers in the region, providing a unique source of DNA for comparing past and present population structure.

With the general goal of comparing the genetic structure before and after human-induced change, we analysed historical and contemporary samples of brown trout at 21 microsatellite loci. We specifically tested the hypothesis that hatchery–wild introgression has led to increased dispersal and resulted in a breakdown of the original genetic structure. Based on theoretical considerations, we then assessed whether wild population declines or admixture with hatchery trout was the most detrimental factor influencing the persistence of adaptive genetic variation.

Materials and methods

Samples and populations

We analysed six anadromous brown trout populations from the Storaa (STO), Skjern (SKJ), Varde (VAR), Sneum (SNE), Kongeaa (KON) and Ribe (RIB) rivers. The localities of the rivers, sample abbreviations, sample sizes and sampling year are shown in Fig. 1. There are no other sizeable rivers harbouring anadromous trout from STO in the north to RIB in the south. However, there have historically been anadromous brown trout populations < 20 km south of

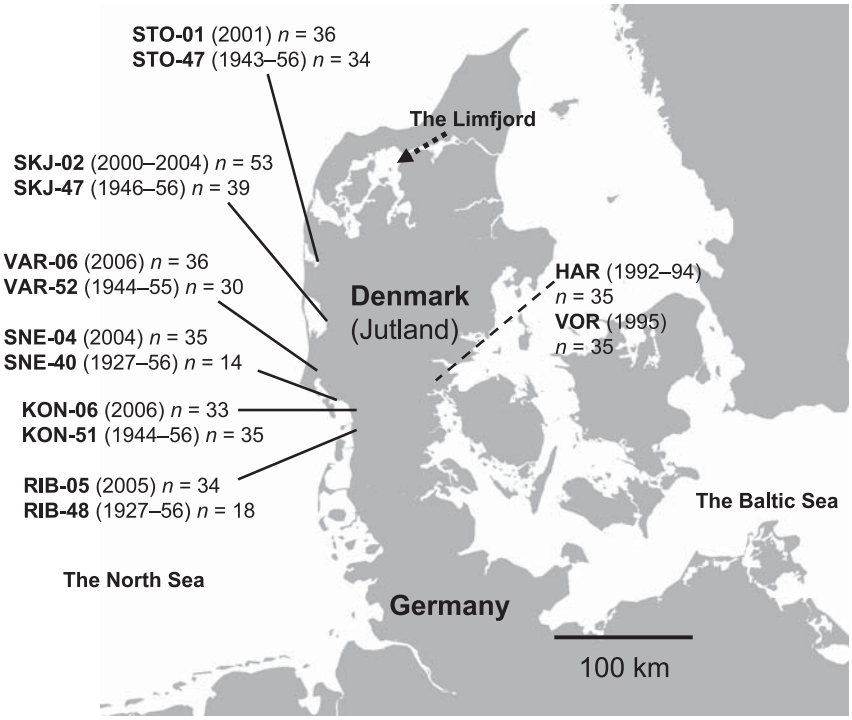

Fig. 1 Map showing the geographical location of the sampled rivers in Jutland, Denmark, along with details on sample abbreviations, sample sizes and year of sampling. Temporal samples from the same populations are denoted by the median year of sampling.

RIB, although natural production in these rivers is now minimal.

Contemporary samples consisted of adipose fin clips of spawning anadromous trout caught in November–January 2000–2006 by electrofishing. Historical samples consisted of dried scales from anadromous trout caught by angling or net fishing in the rivers during spawning runs between 1927 and 1956, but with the majority taken from 1943 to 1956. For some populations, historical sample sizes were low (SNE-40, $N = 14$; RIB-48, $N = 18$), but we attempted to compensate for low statistical power by analysing a relatively high number of loci.

In addition, we analysed samples from the Hårkær (HAR) and Vork (VOR) hatchery strains, which have been used for stocking the rivers (see Fig. 1 for sampling details). Both strains share a partly common history (Hansen *et al*. 2001). VOR was founded in the 1880s by anadromous trout caught in the Kolding River, Eastern Jutland, and HAR was presumably founded by trout from the same river. Exchange of fish between the strains is known to have taken place.

Data on numbers of stocked fish from the two hatchery strains (1992 to the present) were available in the archives of the Technical University of Denmark, National Institute of Aquatic Resources (see Table S1, Supporting information). In some of the rivers, particularly STO and SKJ, the major part of stocking with hatchery strain trout took place prior to 1992. Data prior to 1992 are incomplete, but we obtained data from SKJ dating back to 1977. Sporadic stocking may have taken place in all rivers even before the 1970s, but the numbers of stocked fish are assumed to be low. From the same archives, we also obtained data on the areas of the river systems that could potentially produce trout, in the following denoted 'trout habitat'. This represents the surface areas of the river systems, excluding interspersed lakes, and was used as a proxy for the historically available areas of spawning and nursery habitat (see Table S1).

Molecular analyses

DNA from contemporary samples was extracted using the DNeasy kit (QIAGEN). DNA from historical scale samples was extracted according to Nielsen *et al*. (1999a) with the phenol-chloroform extraction steps replaced by use of the DNeasy kit. To ensure that the analysed loci were unlinked, we analysed 21 microsatellite loci that have been mapped to different linkage groups in the brown trout genome (Gharbi *et al*. 2006): Str60$^{N.}$, Str15$^{V.}$, Str73$^{F.}$ (Estoup *et al*. 1993), SsOsl417$^{F.}$, SsOsl311$^{P.}$ (Slettan *et al*. 1995), SsOsl438$^{V.}$ (Slettan *et al*. 1996), SsOsl32$^{V.}$ (Slettan *et al*. 1997), Ssa85$^{F.}$ (O'Reilly *et al*. 1996), T3–13$^{N.}$ (Estoup *et al*. 1998), Ssa408UoS$^{P.}$ (Cairney *et al*. 2000), Ssa23NVH$^{V.}$, Ssa71NVH$^{P.}$, Ssa52NVH$^{N.}$, Ssa26NVH$^{P.}$, Ssa19NVH$^{V.}$, Str12INRA$^{N.}$, Ssa39NVH$^{P.}$, Ssa64NVH$^{V.}$, Ssa41NVH$^{N.}$, Ssa24NVH$^{P.}$ and Ssa94NVH$^{F.}$ (Gharbi *et al*. 2006). The superscripts $^{F.}$, $^{N.}$, $^{P.}$ and $^{V.}$ denote labelling with the terminal dyes FAM, NED, PET and VIC, respectively. Polymerase chain reaction (PCR) amplification was conducted using the QIAGEN Multiplex PCR Kit (QIAGEN) according to the manufacturer's recommendations and in all cases using an annealing temperature of 57 °C. Four loci with different terminal dyes were amplified

in each multiplex reaction. Several different combinations of loci were used in the multiplex sets; details are available from the authors on request. Amplified loci were analysed on an ABI 3130 Genetic Analyser (Applied Biosystems). Extraction of DNA from historical samples was conducted separately from contemporary samples.

Genetic variation and differentiation

Deviations from Hardy–Weinberg equilibrium (HWE), more specifically significantly lower or higher F_{IS} than expected by chance, were tested by permutation tests implemented in FSTAT 2.9.1. (Goudet 1995). Micro-Checker (Van Oosterhout *et al*. 2004) was used for further analysis of possible null alleles and allelic drop-outs. Genetic variation was quantified by observed (H_O) and expected (H_E) heterozygosities and by allelic richness (El Mousadik & Petit 1996), the latter based on the smallest sample size ($N = 10$, at four of the loci in SNE-40). FSTAT 2.9.1 was also used for these calculations. Genetic differentiation among populations was estimated with θ_{ST} (Weir & Cockerham 1984), with statistical significance being tested by permuting individuals between samples. Moreover, a hierarchical analysis was conducted, estimating differentiation among populations and between temporal samples within populations using Arlequin 3.0 (Excoffier *et al*. 2005). The same software was used for testing for linkage disequilibrium (or rather gametic phase disequilibrium) between all pairs of loci. All tests for HWE, linkage disequilibrium and significance of pairwise θ_{ST} were corrected for multiple tests by applying a false discovery rate (Benjamini & Yekutieli 2001).

Genetic relationships between samples were visualized by a neighbour-joining tree (Saitou & Nei 1987), based on Nei *et al*.'s (1983) unbiased genetic distance. The robustness of the inferred relationships was evaluated by bootstrapping 1000 times over loci. The packages MSA (Dieringer & Schlotterer 2003) and PHYLIP 3.68 (Felsenstein 1989) were used for these analyses.

*Migration (*m*) and effective population size (*N_e*)*

The coalescence-based method Migrate 1.6.5 (Beerli & Felsenstein 2001) was used for estimating the historical interpopulation migration rate M, defined as m/μ, where m denotes migration rate and μ mutation rate, and historical N_e measured as $\theta = 4N_e\mu$. We assumed a stepwise mutation model and based estimates on 15 short (10^4) and five long [10^5 Markov chain Monte Carlo (MCMC) steps] chains. To ensure convergence, we used the 'adaptive heating' option with one 'cold' and three 'hot' chains. Three analyses were conducted, wherein the second and third analysis the point estimates of M and θ from the first and second run, respectively, were used as starting values. As Migrate assumes that demographic parameters have been relatively constant over long time spans, these analyses were only conducted for historical samples.

An assignment test-based method was used for estimating real-time dispersal between populations (Paetkau *et al*. 2004). The local or immigrant status of individuals was assessed by $\Lambda = -\log$ (L_home/L_max), where L_home is the likelihood of the multilocus genotype of the individual in the population in which it was sampled, and L_max is the highest likelihood of the multilocus genotype among all sampled populations. The significance of Λ was assessed using a simulation algorithm (Paetkau *et al*. 2004) with 10^4 simulations and assuming a 1% significance level. GeneClass2 (Piry *et al*. 2004) was used for these calculations, and we conducted the analyses for both sets of historical and contemporary samples. The statistical power for estimating real-time dispersal is a function of numbers of loci, sample size, genetic differentiation, and genetic variation at the loci. Paetkau *et al*. (2004) found that the D_{LR} genetic distance (Paetkau *et al*. 1997) is a better predictor of statistical power than F_{ST}. We therefore calculated D_{LR} for the sets of historical and contemporary samples.

We further estimated N_e using the temporal method *MLNE2* (Wang & Whitlock 2003), which simultaneously estimates N_e (in the following referred to as 'temporal N_e' to separate it from historical N_e) and immigration rate m. The calculations were based on temporal samples (i.e. historical and contemporary samples) from the focal population, whereas the other samples were pooled to represent a source population supplying migrants to the focal population. We conducted two sets of analyses, first by pooling all samples (wild and hatchery strain trout) to represent a source population, and second by using only the two hatchery strain samples as source populations. We also estimated temporal N_e using a method which assumes no gene flow among populations (Wang 2001). We assumed a generation length of 3.5 years (Hansen *et al*. 2002). It should be noted that Wang & Whitlock's (2003) method is considered controversial by some (Waples & Gaggiotti 2006), as the assumed infinite-source migration model is biologically unrealistic. Nevertheless, Wang & Whitlock (2003) found that the method performed well in the case of subdivided populations, as long as the potential source populations could be identified, which was clearly the case in our study. Moreover, both Wang's (2001) and Wang & Whitlock's (2003) methods assume discrete populations, whereas brown trout exhibits overlapping generations. However, given that temporal samples were separated by 15–18 generations, this is not expected to be of major concern for this data set (Waples & Yokota 2007).

Bayesian cluster analysis and admixture proportions

Bayesian clustering implemented in Structure 2.2 (Pritchard *et al*. 2000; Falush *et al*. 2003) was used for estimating the

number of populations/groups represented by the sampled individuals (k) and for estimating individual hatchery–wild admixture proportions without using prior information about the sample of origin. For estimating the most likely k, we conducted runs assuming $k = 1..10$. We assumed an admixture model and correlated allele frequencies. Each run consisted of a burn-in of 10^5 MCMC steps, followed by 5×10^5 steps. Ten replicates were conducted for each k. We plotted the probability of the data [P(D)] and the *ad hoc* statistic Δk (Evanno *et al*. 2005) which measures the steepest increase of the probability of k. Finally, we estimated population-level admixture proportions as the mean of individual admixture in contemporary wild population samples.

Isolation by distance and correspondence between demographic and environmental parameters

Relationships between (i) historical N_e (measured as θ) and areas of trout habitat, (ii) historical and contemporary N_e, and (iii) contemporary N_e and trout habitat were analysed using linear regression. Relationships between genetic distance ($F_{ST}/1 - F_{ST}$ (Rousset 1997) and environmental or demographic distances were analysed by partial Mantel tests (Legendre & Legendre 1998) using the program IBD 1.5 (Bohonak 2002). Environmental and demographic distances for historical samples consisted of geographical distance (shortest waterway distances between river mouths) and pairwise harmonic mean of historical N_e. For contemporary samples, distances included geographical distance, pairwise harmonic mean of temporal N_e, and pairwise differences in admixture proportions of hatchery trout, estimated using Structure (see above). We used pairwise harmonic mean of N_e as a 'distance' measure, as we would expect a negative correlation between N_e and genetic distance due to stronger drift at low N_e.

Results

Contemporary vs. historical genetic variation and differentiation

Genetic variation ranged from four alleles at the loci *Str60* and *Str73* to 42 alleles at *Ssa42NVH*. Allelic richness was similar in historical vs. contemporary wild populations (Table S2, Supporting information). Apart from rare alleles (frequencies < 0.05), there were no indications of loss or appearance of new alleles over time (data not shown). Repeated analysis of subsets of the historical samples (20%) suggested an error rate of 1.7% involving eight loci (*Ssa26NVH*, *Ssa41NVH*, *Ssa94NVH*, *Ssa23NVH*, *SsOSL32*, *Str15*, *Ssa64NVH*, *Ssa408* and *SsOSL438*) and primarily representing allelic drop-outs. However, the true error rate may be underestimated, as failure to amplify an allele in the first round of PCR may be repeated in the second round.

Twenty-two significant deviations from HWE were observed among 294 tests (7.5%) after false discovery rate correction. Four of them involved significantly negative F_{IS} values, i.e. heterozygote excess. They appeared randomly distributed across loci and samples and will not be considered further. The remaining 18 outcomes (6.1%) involved heterozygote deficits (see Table S2), and 12 outcomes were found among the 126 tests involving historical samples (9.5%). Micro-Checker suggested that deviations reflected null alleles, although their occurrence primarily in historical samples would suggest allelic drop-outs. Four significant deviations were observed at *Ssa408* and *SsOSL311* and three at *Ssa71NVH*. Nine of these outcomes involved historical samples. Null allele frequencies estimated using the 'van Oosterhout' algorithm implemented in Micro-Checker (Van Oosterhout *et al*. 2004) ranged from 0 to 0.12 at *Ssa408*, from 0 to 0.08 at *SsOSL311*, and from 0 to 0.14 at *Ssa71NVH*. In a single case, allele frequencies adjusted for the presence of null alleles changed from 0.48 to 0.38 (allele 199 bp at *Ssa71NVH* in STO-47), whereas in the other cases, allele frequencies differed by at most a few per cent. For instance, estimated null allele frequency was 0.12 at *Ssa408* in KON-51, but the largest difference in allele frequency occurred for a 221 bp allele, where adjustment changed the frequency from 0.21 to 0.18. θ_{ST} calculated across all samples was 0.025 (95% CI, 0.020–0.030), whereas omitting *Ssa408*, *SsOSL311* and *Ssa71NVH* yielded almost the same result: 0.024 (95% CI, 0.018–0.030). Moreover, Structure analyses with and without the three loci yielded essentially similar results. We therefore decided to keep these loci in the analyses. Although we cannot exclude artefacts at some of the other loci, we assume that this has had limited influence on the results, particularly because small percentages of artefacts at individual loci would be compensated by the relatively large total number of loci analysed.

Due to low sample sizes, we omitted SNE-40 and RIB-48 from the tests for linkage disequilibria. Among the remaining 2520 tests across populations and loci, 133 (5.3%) were significant ($P < 0.05$) without and 26 (1.0%) significant ($P < 0.05$) after false discovery rate correction (data not shown). There was no obvious tendency towards specific populations or locus pairs being involved in significant outcomes, the latter supporting that all studied loci are indeed unlinked.

Estimates of genetic differentiation (θ_{ST}) among the sets of historical and contemporary samples, respectively, were in both cases 0.018 ($P < 0.001$). However, a hierarchical analysis showed that significant temporal change had occurred; differentiation among populations (F_{CT}) was 0.007 ($P = 0.069$), whereas temporal differentiation within populations (F_{SC}) was higher, i.e. 0.012 ($P < 0.001$). Pairwise θ_{ST} between historical samples ranged from 0.004 to 0.045, with nonsignificant differentiation between samples from the neighbouring populations STO-47 and SKJ-47 and KON-51

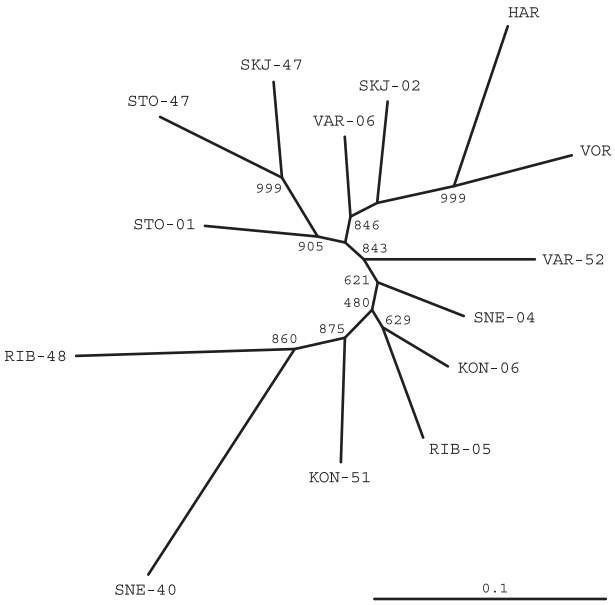

Fig. 2 Neighbour-joining tree based on Nei *et al.*'s (1983) genetic distance. Numbers denote bootstrap support (based on 1000 replicates).

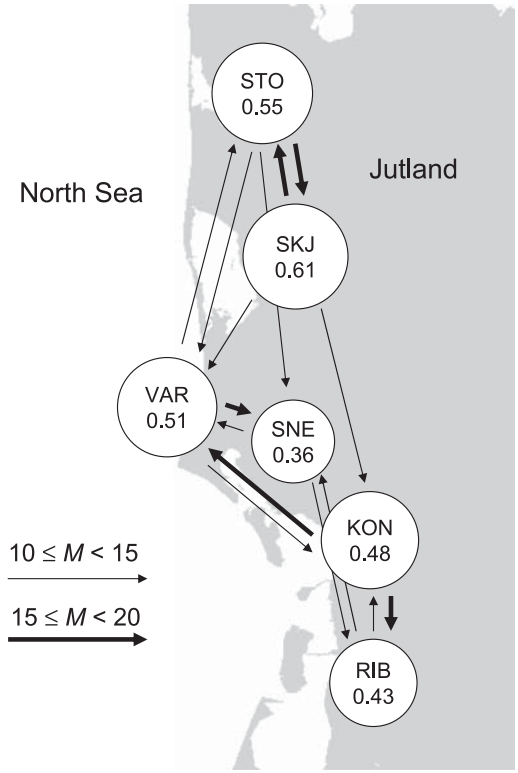

Fig. 3 Map with superimposed historical effective population size (θ) and gene flow estimates (*M*) based on historical samples of brown trout. The direction of gene flow is indicated by arrows. Estimates of $M < 10$ are not shown. The analyses were conducted using the method and software Migrate 2.0.3 (Beerli & Felsenstein 2001).

and RIB-48, respectively (see Table S3, Supporting information). For contemporary samples, estimates of θ_{ST} ranged from 0.001 to 0.063 with nonsignificant differentiation between samples from the neighbouring populations KON-06 and RIB-05, KON-06 and SNE-04 and SNE-04 and VAR-06, respectively. θ_{ST} between the two hatchery strains was moderate (0.022). In most cases, estimates of θ_{ST} were lower between hatchery strains and contemporary samples than between hatchery strains and historical samples (e.g. 0.051 between HAR and SKJ-47 vs. 0.029 between HAR and SKJ-02; 0.034 between VOR and VAR-52 vs. 0.013 between VOR and VAR-06; see Table S3).

The neighbour-joining tree based on Nei *et al.*'s (1983) genetic distance showed that genetic relationships between populations tended to reflect their geographical location (Fig. 2). The historical samples from the three southernmost populations RIB, KON and SNE grouped together with high bootstrap support. The contemporary samples from these populations also showed close relationships and, furthermore, grouped with the three historical samples. Similarly, the historical SKJ and historical and contemporary STO samples formed a group supported by a high bootstrap value. The contemporary samples from SKJ and VAR showed closer relationships to the hatchery strains HAR and VOR than to the historical samples from the same rivers. Collectively, the tree suggested some contingency between historical and contemporary samples in STO, SNE, KON and RIB, whereas significant temporal genetic change had occurred in SKJ and VAR, likely reflecting influence by hatchery strain trout (see below).

Migration and effective population size

The three consecutive runs of Migrate based on historical samples yielded very similar results. We summarize the outcome of the third analysis in Fig. 3 and provide median and 95% confidence intervals for all *M* and θ values in Table S4, Supporting information. The analysis suggested a genetic structure characterized by populations ranging in size from $\theta = 0.36$ (SNE) to $\theta = 0.61$ (SKJ), gene flow occurring primarily between geographically proximate populations and without indications of strongly asymmetric gene flow.

The mean D_{LR} distance (Paetkau *et al*. 1997) between populations was 6.0 for historical and 5.5 for contemporary samples. This is above the recommended value of 3, indicating sufficient statistical power for estimating dispersal (Paetkau *et al*. 2004). However, this recommendation concerns dispersal between pairs of populations; it is more difficult to interpret power when multiple populations are involved, and furthermore, D_{LR} varied among specific pairs of samples. Nevertheless, the relative similarity of D_{LR} for historical and contemporary samples suggests that a relative comparison of dispersal across time periods is valid, whereas specific dispersal rates should be interpreted with

Focal population	Gene flow	N_e (95% CI)	m (95% CI)
STO	None	819 (524–1605)	NA
	All	299 (189–500)	0.037 (0.022–0.059)
	Hatchery	265 (186–388)	0.017 (0.011–0.026)
SKJ	None	327 (262–418)	NA
	All	133 (91–193)	0.081 (0.054–0.123)
	Hatchery	145 (107–197)	0.039 (0.027–0.056)
VAR	None	579 (387–1014)	NA
	All	224 (55–452)	0.102 (0.051–0.567)
	Hatchery	221 (144–376)	0.035 (0.021–0.054)
SNE	None	1551 (690– >4000)	NA
	All	417 (175–1188)	0.046 (0.017–0.111)
	Hatchery	377 (221–745)	0.016 (0.007–0.027)
KON	None	866 (537–1846)	NA
	All	423 (241–725)	0.030 (0.018–0.052)
	Hatchery	280 (197–427)	0.015 (0.009–0.022)
RIB	None	1695 (703– >4000)	NA
	All	587 (298–1980)	0.013 (0.004–0.026)
	Hatchery	355 (230–585)	0.008 (0.007–0.013)

Table 1 Estimates of temporal effective population size (N_e) using Wang's (2001) temporal method assuming no gene flow, and N_e and immigration rate (m) using the temporal method by Wang & Whitlock (2003). Two sets of analyses were conducted using the latter method, one assuming immigration from all populations ('All') and one assuming immigration only from hatchery strain trout ('Hatchery'). NA, not applicable

caution. In historical samples, 21 putative first-generation migrants were suggested among 171 individuals, yielding an estimated mean immigration rate across populations of 0.12 (SD 0.05). In contemporary samples, only a single individual was suggested to be a first-generation immigrant from a hatchery strain, reflecting that stocking with the strains had ceased at the time of sampling. Seventeen putative wild immigrants were suggested among the 227 individuals from contemporary wild samples, and the estimated mean immigration rate across populations was 0.07 (SD 0.03). Hence, there was no evidence to support increased dispersal as a result of recent introgression by hatchery strain trout. In fact, it could be argued that dispersal had decreased over time, although the standard deviations of the estimates do not allow for firm conclusions.

N_e estimates based on a temporal method assuming a closed population (Wang 2001) ranged from 327 (SKJ) to 1695 (RIB; see Table 1). Application of a method taking gene flow into account (Wang & Whitlock 2003) and assuming a pooled source population encompassing wild populations and hatchery strains yielded lower N_e estimates, ranging from 133 (SKJ) to 587 (RIB). It is noteworthy that the relative magnitude of temporal N_e among populations was different from historical N_e (θ). For instance, SKJ was suggested to have the largest historical N_e, whereas temporal estimates suggested SKJ to have the smallest N_e.

Estimates of immigration rate (m) with Wang & Whitlock's (2003) method ranged from 0.013 (RIB) to 0.102 (VAR) (Table 1). Finally, assuming that the hatchery strains were the only sources of gene flow yielded relatively similar N_e estimates but lower m estimates, ranging from 0.008 (RIB) to 0.039 (SKJ). This strongly suggests that gene flow from hatchery strains has occurred, but also shows that wild–wild gene flow has been more important over the encompassed time span (Table 1).

Bayesian cluster analysis and admixture proportions

The Structure analyses provided strongest support for $k = 3$, both when considering the probability of the data [P(D)] and the *ad hoc* statistic Δk (Evanno *et al*. 2005) (see Fig. S1, Supporting information). The distribution of individual admixture proportions showed that cluster 1 corresponded to the hatchery strains HAR and VOR (Fig. 4). Cluster 2 corresponded to the historical samples from STO and SKJ (STO-47 and SKJ-47), whereas cluster 3 corresponded to the historical SNE, KON and RIB samples (SNE-40, KON-51 and RIB-48). The geographically intermediate VAR-52 sample showed a mixture of clusters 2 and 3. Analyses assuming $k > 3$ did not resolve further groupings of individuals. Instead, additional clusters were 'admixed' across individuals from multiple populations. Although the two hatchery strains HAR and VOR could not be distinguished in the full Structure analysis, separate analysis of the samples resolved them into two clusters (data not shown). However, analysis of subsets of the wild populations did not resolve further groups.

The continuous distribution of clusters 2 and 3 in the wild historical samples and the lack of ability to resolve further groupings despite significant θ_{ST} values reflect well-known problems with Structure for handling low differentiation and isolation by distance (Falush *et al*. 2003; Waples & Gaggiotti 2006). Nevertheless, individual admixture proportions in the historical samples proved highly informative for evaluating temporal genetic change. The distribution of clusters 2 and 3 was retained in all contemporary samples

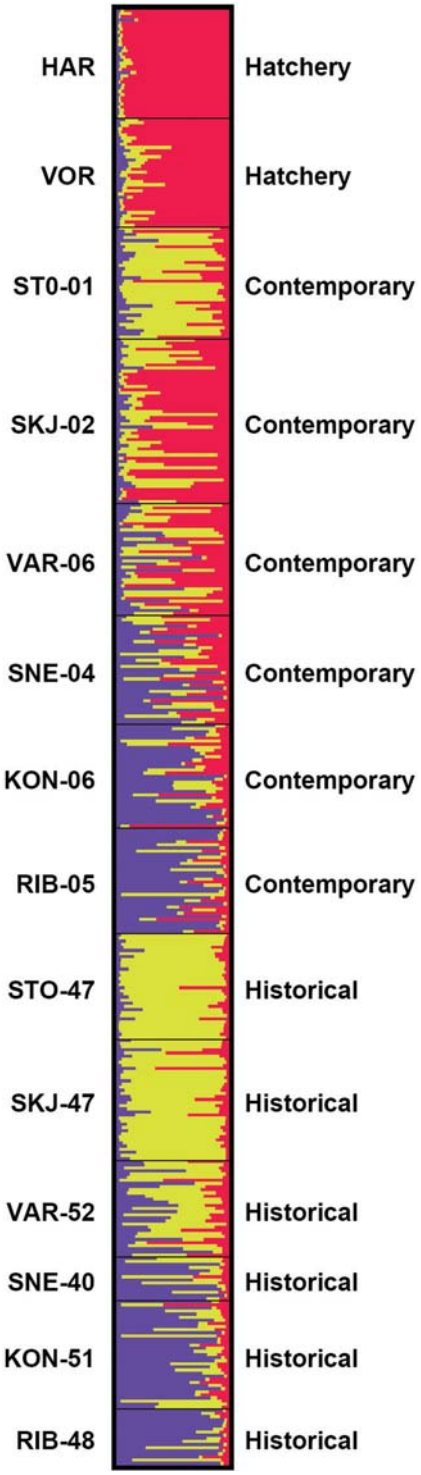

Fig. 4 Bayesian clustering of all individuals using Structure 2.0 (Pritchard *et al*. 2000; Falush *et al*. 2003), assuming three different clusters of individuals ($k = 3$), an admixture model and no prior population information. Each horizontal bar denotes an individual, and the three colours denote the different inferred clusters. See Fig. 1 for sample abbreviations

from STO-01 in the north to RIB-05 in the south, but with a significant component of cluster 1 (hatchery strain trout) superimposed, particularly in SKJ-02 and VAR-06, whereas KON-06 and RIB-05 appeared considerably less admixed. The differences in genetic contribution by hatchery strain trout were further evident by considering population-level estimates of admixture proportions: 0.22 (STO-01), 0.64 (SKJ-02), 0.45 (VAR-06), 0.31 (SNE-04), 0.19 (KON-06) and 0.14 (RIB-05).

Isolation by distance and correspondence between demographic and environmental parameters

Linear regression showed a significant positive correlation between trout habitat (A in km^2) and historical N_e (θ): $\theta = 0.10\ A + 0.37$ ($r^2 = 0.72$; $F_4 = 10.2$, $P = 0.033$). However, the nonzero y-intercept suggests a more complex relationship than merely linear. Correlations between θ and temporal N_e and between temporal N_e and trout habitat area were negative and nonsignificant (data not shown).

Partial Mantel tests for the historical samples showed a significant association between genetic and geographical distance, also when controlling for historical effective population size (θ). Moreover, there was a significant negative association between genetic distance and pairwise harmonic mean of θ when controlling for geographical distance (Table 2). Significant isolation by distance was also observed among the contemporary samples, but there was no significant association between genetic distance and pairwise harmonic mean of temporal effective population size (N_e). However, a significant, positive association was observed between genetic distance between populations and difference in admixture proportions of hatchery strain trout (Table 2).

Discussion

Admixture with hatchery strain trout

The rivers along the Jutland west coast represent some of the most intensively stocked brown trout populations in Denmark. Hence, it is not surprising that wild populations showed significant, albeit varying degrees of admixture with hatchery trout. It has been suggested that introgression due to stocking depends on the immigration (m)–selection (s) balance; if $m > s$ then immigration overrides selection and strong introgression may occur despite lower fitness of hatchery fish relative to wild fish (Hansen 2002). Moreover, it is possible that s against hatchery fish could in itself vary with population size, with stronger selection acting at higher densities of wild fish. For instance, in Atlantic salmon, it has been suggested that small precocious males of hatchery origin may be an important vehicle causing introgression into wild populations (Garant *et al*. 2003). It can be envisaged

Table 2 Partial Mantel tests for association between genetic differentiation, geographical distance and indicator variables. The indicator variables include (i) harmonic mean of historical effective population size (θ) in historical samples, (ii) pairwise harmonic mean of effective population size (N_e) in contemporary samples, and (iii) pairwise differences in admixture proportions of hatchery strain trout between contemporary samples. Correlation coefficients, *r*, and *p* values were determined by 10 000 permutations. The software IBD 1.5 (Bohonak 2002) was used for the analyses

	Historical samples			
	Pairwise harmonic mean θ			
	r	*p*		
Genetic and indicator	0.145	0.347		
– controlling for geographical	–0.634	0.010		
Genetic and geographical	0.549	0.005		
– controlling for indicator	0.760	0.006		
	Contemporary samples			
	Pairwise harmonic mean N_e		Difference in admixture proportions between samples	
	r	*p*	*r*	*p*
Genetic and indicator	–0.421	0.141	0.673	0.015
– controlling for geographical	–0.105	0.436	0.639	0.022
Genetic and geographical	0.529	0.042	0.529	0.042
– controlling for indicator	0.367	0.159	0.471	0.089

that such precocious hatchery males employing sneaking tactics may have high reproductive success at low densities of wild precocious and adult males, but lower success in breeding competition at higher densities of wild males.

As the records on numbers of stocked trout are incomplete prior to 1992, we are unable to estimate the immigration rate of stocked fish and relate it to estimated admixture proportions. However, we note that the two populations showing the lowest levels of admixture, KON and RIB (hatchery trout admixture proportions of 0.19 and 0.14, respectively), also show the highest temporal N_e (see Table 1). Conversely, the two populations showing the lowest temporal N_e, SKJ and VAR, are also the most strongly admixed (admixture proportions of 0.64 and 0.45, respectively). It is not possible to distinguish between the possibilities that (i) smaller populations are more prone to admixture with hatchery strain fish or (ii) populations that are strongly admixed experience population declines due to the genetic load imposed by stocked fish (Lynch & O'Hely 2001). However, the results suggest important interactions between admixture rate and effective population size.

Population declines and effective population size

It is evident from census population size (*N*) estimates and catch statistics that the studied trout populations have declined considerably (Dieperink *et al*. 1997), but does this translate into a decline of N_e? Indeed, the process of genetic compensation, wherein either N_e is not reduced proportionately to *N* as *N* declines, possibly due to increased contribution by resident fish, has been suggested in several salmonid fishes (Ardren & Kapuscinski 2003; Araki *et al*. 2007; Fraser *et al*. 2007b).

Our results do not allow for directly demonstrating declines of N_e. In principle, N_e could be estimated using a single-sample approach, such as a linkage disequilibrium (LD) method (e.g. Waples & Do 2008), which would provide a direct comparison of N_e in historical and contemporary samples. However, both drift and admixture contributes to linkage (or gametic phase) disequilibria, whereas in the LD method, all disequilibria are ascribed to drift (Waples 2006). Hence, the validity of applying the LD method to the strongly admixed contemporary samples (up to 64% in SKJ-02) is questionable.

θ values estimated using Migrate could also be directly transformed to historical N_e values by assuming a specific microsatellite mutation rate and then subsequently comparing these values to temporal N_e estimates. However, assuming realistic mutation rates in the range of 10^{-4}–10^{-3} could lead to conclusions of both recent population decline and expansion, depending on the specific mutation rate assumed. Nevertheless, an indirect comparison of historical and temporal N_e suggests considerable decline in some populations. First, there is no positive correlation between historical (θ) and temporal N_e among the total set of populations. Second, STO, SKJ and VAR are suggested to be the historically largest

populations with θ values ranging from 0.51 to 0.61 (Fig. 3). Yet, using Wang & Whitlock's (2003) method, these populations are suggested to exhibit the smallest temporal N_e, ranging between 133 and 299 (Table 1). RIB is the population assumed to have been least affected by habitat degradation and subsequent population declines. If we tentatively assume that the temporal N_e estimate in RIB (587) is roughly representative of N_e in other populations prior to the 1950s, then this would indeed suggest that N_e has declined significantly in STO, SKJ and VAR.

Historical and contemporary genetic structure

The historical genetic structure is characterized by N_e reflecting the amount of trout-producing areas (trout habitat) within river systems, isolation by distance and high dispersal rates, although as stated previously, the results obtained using Paetkau *et al*.'s (2004) method should be interpreted with caution. This type of regional structure may be common in a number of salmonid species, where connectivity among rivers is not impeded by physical barriers, as observed in population systems of Atlantic salmon (Nielsen *et al*. 1999b; Dionne *et al*. 2008) and rainbow trout (*Oncorhynchus mykiss*) (Narum *et al*. 2008). Conversely, however, other studies of population systems encompassing species like Atlantic salmon, brown trout and brook charr (*Salvelinus fontinalis*) have estimated asymmetric gene flow from larger to smaller populations (Fraser *et al*. 2004, 2007b; Hansen *et al*. 2007; Palstra *et al*. 2007). This is not evident in the present study (see Fig. 3, which suggests relatively equal gene flow between neighbouring populations), but may simply reflect that differences in historical N_e are too small to leave a detectable signature of asymmetric gene flow. Nevertheless, the partial Mantel tests (Table 2) suggest an effect of historical N_e (θ) on pairwise genetic differentiation. The association is negative, and we ascribe it to stronger drift in the smaller populations leading to increased differentiation.

Although genetic differentiation per se, measured as θ_{ST}, is similar among the sets of historical and contemporary populations, the contemporary genetic structure nevertheless differs from the historical structure. Considerable temporal genetic divergence has occurred within populations, as evidenced by the hierarchical spatio-temporal analysis of genetic differentiation. There is no association between historical and temporal effective population size, and between temporal effective population size and the areas of trout habitat within rivers. Although significant isolation by distance has been retained in the contemporary samples, different admixture proportions of hatchery trout also influence the current genetic structure, as evidenced by the partial Mantel tests (Table 2).

Does the change to population genetic differentiation mean that the genetic structure has broken down completely? We argue that it has not. The estimate of dispersal rate using Paetkau *et al*.'s (2004) approach was 0.12 (SD 0.05) and comparable to the estimate of 0.07 (SD 0.03) based on contemporary samples. Gene flow does not equal dispersal; for instance, dispersal may be higher than gene flow if immigrants exhibit lowered fitness in a foreign environment (Garant *et al*. 2007). However, assuming dispersal and gene flow are correlated, then the relative similarity of dispersal rates estimated for historical and contemporary populations suggests that gene flow has not increased; if anything, there is a trend towards decreased gene flow, possibly due to population declines. Moreover, the neighbour-joining tree (Fig. 2) illustrates that the geographical location of populations is reflected in their genetic relationships. These relationships are to a considerable extent maintained over time, although significantly influenced by admixture with hatchery strain trout. Finally, despite limitations of the Structure analysis, the wild populations are represented by two clusters showing a distinct geographical distribution from north to south in the historical samples. After accounting for a third cluster representing hatchery strain trout, it is evident that the historical distribution of these two clusters has been retained in the contemporary samples (Fig. 4).

Collectively, we conclude that N_e has changed significantly over time within some populations, as has the genetic composition *within* populations due to admixture with stocked hatchery trout. However, we reject the hypothesis that dispersal and/or gene flow rates have increased as a result of introgression by hatchery trout. Moreover, stocking has not completely obliterated the original genetic structure *between* populations. Indeed, our analyses suggest that the original genetic structure between populations is still evident after accounting for hatchery–wild admixture.

Conservation implications of population decline and admixture

The census population size declines have clearly affected productivity of our study populations negatively; but even the smallest temporal N_e estimates exceed 100, suggesting that problems concerning accumulation of inbreeding should not be a major concern (the '50-rule'; Franklin 1980). It should be noted, however, that the temporal estimates represent the harmonic mean of N_e over the entire sampling period. Contemporary N_e of the populations may therefore be substantially lower and could compromise the future fitness of populations if N_e remains low.

Reduced N_e could also have negative consequences for the persistence of local adaptation in the face of gene flow. Notably, local adaptation ultimately reflects the interplay between selection, gene flow and genetic drift (Kawecki & Ebert 2004). Under a stepping-stone model of population structure, the potential for local adaptation can be roughly evaluated using the approach by Adkison (1995). If we assume that N_e was 500 and m 0.02 prior to recent population

declines (roughly equivalent to the parameters estimated for the least disturbed population RIB; Table 1) and that selection regimes are similar across all six populations, then using Adkison's (1995) approach, local adaptation is predicted even for selection coefficients (s) as low as 0.001. A similar calculation assuming that N_e on average has decreased from 500 to 275 [the harmonic mean of N_e estimates of individual populations using Wang & Whitlock's (2003) method; Table 1] yields essentially the same result, i.e. potential for local adaptation even at $s = 0.001$. This assumes that local adaptation occurs primarily on a regional scale, which is indirectly supported by the fact that the rivers are geomorphologically quite similar and all flow into the North Sea. If we instead assume that local adaptation occurs at the scale of individual rivers, then at N_e values of both 500 and 133 (as estimated for SKJ) and m of 0.02, the potential for local adaptation is constrained by m, not N_e. Hence, population declines are not likely to have significantly affected the persistence of local adaptation in the studied populations.

Whereas we assume that population declines have had a limited influence on adaptive variation, introgression by stocked hatchery strain trout could have significant negative consequences. In some of the populations approximately half of the contemporary gene pool is derived from hatchery trout, which raises concerns both about swamping of indigenous adaptive variation imposing a genetic load on the wild populations (Lynch & O'Hely 2001) and outbreeding depression. The results of this study do not allow for inferring outbreeding depression. However, this phenomenon has previously been demonstrated in experiments with both Atlantic salmon and pink salmon (*Oncorhynchus gorbuscha*) simulating population translocations and hatchery–wild fish interactions (McGinnity *et al*. 2003; Wang *et al*. 2007). Moreover, experiments on artificially selected Atlantic salmon at the gene expression level suggest disruption of non–additive gene interactions through hatchery–wild mixing (Roberge *et al*. 2008). Nevertheless, outbreeding depression is not necessarily a given outcome of interbreeding between wild and farmed fish (Fraser *et al*. 2008), and in general, our knowledge of the persistence and duration of outbreeding depression is incomplete (Edmands 2007).

Conclusions

Analysis of samples covering a time span of more than 60 years proved highly informative for resolving the genetic structure of a population system in a relatively natural state, and for assessing how recent population declines and stocking have affected the contemporary genetic structure. Despite strong anthropogenic disturbance, the original genetic structure was to a considerable extent still evident in the contemporary populations. Admixture with hatchery strain trout was the single most important factor causing changes to the contemporary genetic structure and possibly also compromising adaptive variation. However, our results also suggested important interactions between population declines and admixture.

The studied stocking scenario is of relevance for many other cases of fish species experiencing spawning intrusion from stocked or escaped farmed fish (Hutchings & Fraser 2008), along with cases of gene flow from captive to wild populations in other organisms, ranging from plants to mammals (Ellstrand *et al*. 1999; Verardi *et al*. 2006). To date, most studies have focused on quantifying the problems and analysing the involved mechanisms, with less focus on the conservation prospects of the many populations that have now become admixed (but see Allendorf *et al*. 2001). Our study raises a complementary set of questions of relevance for future conservation efforts. To what extent does the original genetic composition and population structure still persist after admixture? Are adverse effects of admixture on adaptive variation reversible or irreversible? And, crucially, what is the conservation value of admixed populations? Our results demonstrate the usefulness of spatio-temporal analysis of neutral markers for answering the first of these questions. However, answering the other two questions will require improving our understanding of the genetic architecture of adaptive traits and how traits are affected by population admixture, along with integration of results into frameworks for setting conservation priorities.

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Michael Hansen's research focus involves characterizing genetic population structure and the evolution of local adaptations in freshwater and marine fishes. He is particularly interested in analyzing the temporal dimension of neutral and adaptive processes using historical and contemporary samples. Dylan Fraser's current research involves the application of genetic and evolutionary principles to biodiversity conservation and fisheries management. Kristian Meier is a PhD student at the Technical University of Denmark, National Institute of Aquatic Resources. His research focuses on analyzing local adaptation in salmonid fishes at the genomic and transcriptomic levels. Karen-Lise Mensberg is a lab technician, in charge of the molecular laboratory work.

Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1 Probability of the data set representing 1..10 clusters [P(D)], as determined by replicate analyses using STRUCTURE 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003) (black points +/− s.d.), and the ad hoc statistic Δk, measuring the steepness of increase of P(D) (Evanno *et al.* 2005) (red line).

Table S1 Data on the surface areas of potentially trout producing parts of the six studied river systems, along with information on the number of stocked trout from the two hatchery strains HAR (Hårkær) and VOR (Vork).

Table S2 Summary of total number of observed alleles per locus, allele size ranges for the loci, allelic richness (AR) based on the smallest sample size in the data set, i.e. N = 10 for four loci in SNE-40, outcome of tests for deviations from expected Hardy-Weinberg proportions (HWE) with heterozygote excess as alternative hypothesis, expected (H_e) and observed heterozygosity (H_o), and sample sizes (N) of the studied populations

Table S3 θ_{ST} between all pairs of samples, along with tests of their significance.

Table S4 Scaled estimates of historical effective population size (ϑ; values in diagonal and denoted by bold) and migration rate between populations (M) along with their 95% confidence intervals, estimated using MIGRATE 2.0.3 (Beerli & Felsenstein 2001).

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