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Microsatellite and mitochondrial DNA polymorphism reveals life-history dependent interbreeding between hatchery and wild brown trout (*Salmo trutta* L.)

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Abstract

The effects of stocking hatchery trout into wild populations were studied in a Danish river, using microsatellite and mitochondrial DNA (mtDNA) markers. Baseline samples were taken from hatchery trout and wild trout assumed to be unaffected by previous stocking. Also, samples were taken from resident and sea trout from a stocked section of the river. Genetic differentiation between the hatchery strain and the local wild population was modest (microsatellite F_{ST} = 0.06). Using assignment tests, more than 90% of individuals from the baseline samples were classified correctly. Assignment tests involving samples from the stocked river section suggested that the contribution by hatchery trout was low among sea trout (< 7%), but high (46%) among resident trout. Hybrid index analysis and a high percentage of mtDNA haplotypes specific to indigenous trout observed among resident trout that were assigned to the hatchery strain suggested that interbreeding took place between hatchery and wild trout. The latter result also indicated that male hatchery trout contributed more to interbreeding than females. We suggest that stronger selection acts against stocked hatchery trout that become anadromous compared to hatchery trout that become resident. As most resident trout are males this could also explain why gene flow from hatchery to wild trout appeared to be male biased. The results show that even despite modest differentiation at neutral loci domesticated trout may still perform worse than local populations and it is important to be aware of differential survival and reproductive success both between life-history types and between sexes.

Keywords: assignment test, gene flow, microsatellite, mitochondrial DNA, *Salmo trutta*, stocking

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Introduction

The advent of molecular markers, in particular microsatellite DNA, is fuelling the development of numerous new research possibilities in population and conservation genetics (Estoup & Angers 1998; Beaumont & Bruford 1999), including the development of new statistical techniques that allow identification of immigrants and their descendants (Rannala & Mountain 1997; Waser & Strobeck 1998), a subject of critical importance for monitoring gene flow or detecting introgression. Genetic interactions between wild and domesticated fish (Hindar *et al*. 1991a) is one area of conservation genetics where microsatellite markers are expected to be particularly useful, as in many instances the limited resolution power of traditionally used markers, such as allozymes and mitochondrial DNA (mtDNA), has not allowed for the detection of interbreeding and introgression and for the tracing of the fate of stocked fish in the wild.

Many important insights into the dynamics of gene flow from domesticated fish into wild fish populations have been gained using allozyme markers (Chilcote *et al*. 1986; Taggart & Ferguson 1986; Largiadèr & Scholl 1996). However, in most cases the resolution power of allozymes has only allowed the genetic discrimination of domesticated and wild populations that are genetically strongly divergent, such as those derived from different phylogeographic lineages (Largiadèr & Scholl 1996). In cases where hatchery strains have been founded from populations in

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the same geographical region as the stocked populations, genetic divergence at allozymes is usually low. At the same time, however, domestication and different selection regimes in hatcheries compared to the wild may have strongly affected traits and loci subject to selection (e.g. Ruzzante 1994; Fleming & Einum 1997). Therefore, there is a considerable need and interest in examining potential genetic interactions between wild and domesticated populations that are only moderately differentiated at the level of presumably neutral loci. Differentiation is expected to be stronger at the mtDNA level, resulting from the smaller effective number and stronger drift compared to nuclear loci (Birky *et al*. 1989). However, even though some studies have demonstrated the utility of mtDNA analysis for the study of genetic interactions between wild and domesticated salmonids (e.g. Danzmann & Ihssen 1995; Hansen *et al*. 1995) mtDNA only traces female gene flow and inferences are drawn on the basis of just one haploid marker locus. In contrast, a variable number of microsatellite DNA loci can be screened and considerable information can be gained from individual multilocus genotypes. This information can then be used in assignment tests (Paetkau *et al*. 1995; Waser & Strobeck 1998) and related procedures (Rannala & Mountain 1997) for classifying individuals into their population of origin even when genetic differentiation among populations is relatively weak.

Our goal in the present study was to obtain a deeper insight into the dynamics of gene flow from stocked hatchery trout into wild trout populations. More specifically, we examined whether there was a relationship between the various life-history forms of stocked trout, i.e. anadromous and resident, and their ability to interbreed with wild trout. We assessed the utility of polymorphism at seven microsatellite loci, combined with variation in mtDNA, to detect interbreeding between wild and stocked domesticated brown trout (*Salmo trutta* L.) in the Karup River, Denmark. We used anadromous indigenous trout and trout from the hatchery strain used for stocking as baseline samples. We then analysed samples of resident and anadromous trout (returning sea trout) collected in a stocked section of the river. Using assignment tests and a hybrid index we demonstrated the near absence of hatchery trout or descendants from hatchery trout among anadromous trout. In contrast, there was a significant component of hatchery trout among the resident trout, and there were indications of male-biased gene flow from hatchery to wild trout.

Materials and methods

Studied localities

We focused on the Karup River, Denmark (see Fig. 1), which presently contains a large population of brown trout. As in most other Danish rivers the population

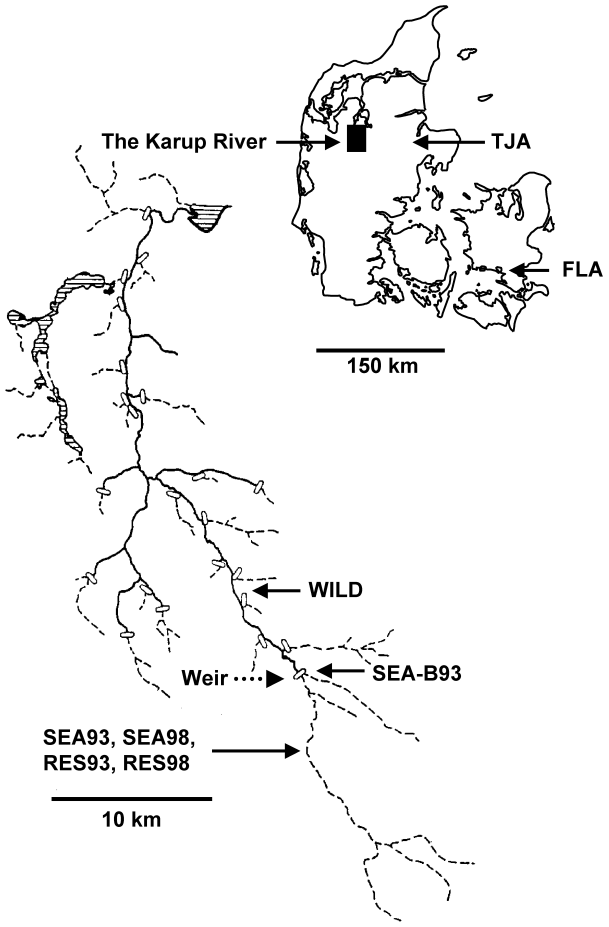

Fig. 1 Map showing the approximate location of sampling localities in Denmark and in the Karup River system.

consists of a mixture of resident and anadromous trout (sea trout). Resident trout spend their entire life cycle within the same river system, whereas anadromous trout migrate to forage in the sea but return to their natal river to spawn (Elliott 1994). During the 1980s the Karup River system was stocked with several hundreds of thousands of hatchery trout; however, mtDNA and microsatellite analyses showed that these fish had not made a detectable genetic contribution to the present population (Hansen *et al*. 1995, 2000). From 1990, stocking of hatchery trout was replaced by supportive breeding, i.e. stocking of offspring of indigenous wild trout, but in the upper part of the main river stocking with hatchery trout [annually 4000–10 000 fry plus an unknown number of larger (> 25 cm, age 2–3+) resident trout], along with stocking of local trout, took place until 1997. Even though natural reproduction also takes place in this part of the river it is thought to be small compared to reproduction in the lower part of the river, as much less suitable spawning area is available. The upper part of the main river is partly isolated from the lower part by a weir built in connection with a rainbow trout (*Oncorhynchus mykiss*) fish farm (see Fig. 1). Ascending

Table 1 Details of samples, sample abbreviations, status of samples (wild or hatchery trout, anadromous or resident), year of sampling and sample sizes

Sample	Sample abbreviation	Status of sample	Year of sampling	Sample size (microsatellites/ mtDNA)
Hårkær Hatchery, baseline sample	HAT	Hatchery strain	1993	49/49
Karup River, baseline sample	WILD	Wild, unaffected by hatchery trout, anadromous	1993	50/50
Karup River, resident trout above barrier	RES93	Wild, stocked with hatchery trout, resident	1993	41/41
Karup River, resident trout above barrier	RES98	Wild, stocked with hatchery trout, resident	1998	15/15
Karup River, sea trout above barrier	SEA93	Wild, stocked with hatchery trout, anadromous	1993	13/13
Karup River, sea trout above barrier	SEA98	Wild, stocked with hatchery trout, anadromous	1998	18/18
Karup River, sea trout below barrier	SEA-B93	Wild, stocked with hatchery trout, anadromous	1993	25/25
Tjærbæk River	TJA	Wild, nonstocked, anadromous	1994	44/51
Flads River	FLA	Wild, nonstocked, anadromous	1997	58/59

sea trout wait below the weir and are only able to pass it during high water levels.

Details of the samples, including sample locality abbreviations, are listed in Table 1. The Hårkær hatchery strain (HAT), which was used for stocking the Karup River, was sampled in 1993. This strain is assumed to be of a mixed origin, but primarily founded from sea trout populations from rivers in southern Jutland (with a waterway distance approximately 200–300 km from the Karup River; details on the history of the strain are given in Hansen *et al*. 1997). In the same year (in November) a sample was taken of sea trout spawners (WILD) from 4 to 10 km downstream from the isolated part of the Karup River. Based on the results of previous studies (Hansen *et al*. 1995, 2000) we assumed that trout from this part of the river are probably genetically unaffected by stocked hatchery trout. In November 1993 one sample of sea trout spawners was taken immediately below the weir (SEA-B93). We assumed that these fish were waiting to ascend the upstream part of the river. Further, both in November 1993 and December 1998 samples were taken of both resident trout (RES93 and RES98) and sea trout (SEA93 and SEA98) that had been able to ascend the upstream part of the river. Sea trout and resident trout could be distinguished based on colouration patterns. To exclude trout from the samples of resident trout that might later smoltify and become sea trout we only included trout larger than 20 cm (beyond the typical size of smolts in Danish rivers [12–15 cm; Rasmussen 1986]). Finally, we included samples from two other Danish rivers, the Tjærbæk (TJA) and Flads (FLA) Rivers, as 'outgroup samples' for the assignment analyses (see below). All samples of wild trout were taken by electrofishing.

Microsatellite and mtDNA analyses

DNA was extracted from muscle or adipose fin tissue stored in ethanol, using the phenol–chloroform extraction protocol by Taggart *et al*. (1992). One tetranucleotide and six dinucleotide microsatellite loci were analysed: *Str* 15 and *Str* 60 (Estoup *et al*. 1993), *Ssa* 85, *Ssa* 197 (tetranucleotide microsatellite) (O'Reilly *et al*. 1996), *SsoSL* 417, *SsoSL* 438 (Slettan *et al*. 1995) and *Ss*Hae*III.14.20* (Goodier unpublished, GenBank accession number U10050). One primer of each primer set was end-labelled with the fluorescent dye CY5, and the microsatellites were analysed on a Pharmacia ALFexpress automated sequencer. In order to facilitate scoring of highly variable loci, individuals with known genotypes were included in all gel runs and in some cases the same individuals were analysed twice in order to verify the reproducibility of results. Gels were scored using the program ALLELINKS version 1.00 (Pharmacia).

For analysing mtDNA variability we PCR amplified the ND-1 and ND-5/6 coding regions using primers described by Nielsen *et al*. (1998). The ND-1 segment was analysed with the restriction endonucleases *Ava*II, *Alu*I, *Hae*III, *Hin*fI, *Hpa*II and *Dde*I, whereas the ND-5/6 segment was analysed with *Ava*II, *Hae*III, *Hin*fI, *Taq*I and *Xba*I. For further technical details, including PCR, restriction digest and gel run conditions, see Hansen *et al*. (1997) and Nielsen *et al*. (1998).

Statistical analyses

Departure from Hardy–Weinberg equilibrium at the microsatellite loci was tested by exact tests using GENEPOP 3.1c (Raymond & Rousset 1995a). Genetic differentiation

between samples at the level of microsatellites was analysed by calculating pairwise F_{ST} values. Their significance was tested by permuting individuals between samples. In addition, exact tests for differences in allele frequencies (Raymond & Rousset 1995b) were performed and single locus probabilities were combined over loci by Fisher's method. The programs GENEPOP 3.1c and ARLEQUIN 1.1 (Schneider *et al*. 1997) were used for these analyses. Differentiation at the mtDNA level was tested by adding a 'dummy allele' to the haplotypes and using the test for genotypic differentiation included in GENEPOP.

The genetic relationships among samples were also examined using multidimensional scaling analysis (MDSA) of the matrix of D_A (Nei *et al*. 1983) pairwise genetic distances. We used Splus© standard code for this analysis and scaling components 1 vs. 2, 1 vs. 3, 1 vs. 4, and 2 vs. 3 were plotted. MDSA plots allow representation of the genetic relationship among samples with minimum loss of information and in more dimensions than the two allowed by phylogenetic trees. This procedure will therefore probably describe data more truthfully than trees when there is considerable genetic exchange between and among close geographical neighbours (Cavalli-Sforza *et al*. 1994) as is probably the case in our study.

For classifying individuals into groups of wild or hatchery trout we used assignment tests as defined by Paetkau *et al*. (1995); however, probabilities of individuals belonging to populations were calculated using a Bayesian approach following Rannala & Mountain (1997). These analyses were performed using the program GENECLASS 1.0 (Cornuet *et al*. 1999; http://www.ensam.inra.fr/URLB/geneclass/geneclass.html).

Briefly, for each individual multilocus genotype the marginal probability in each of the populations is calculated, based on the allele frequencies of the samples, and an individual is assigned to the sample in which it has the highest marginal probability. We used the sample of trout from the nonstocked part of the Karup River (WILD) and from the Hårkær Hatchery strain (HAT) as baseline samples and then assigned individuals from samples from the stocked section of the river to these two baseline samples. A potential problem with this procedure is that all individuals are assigned to one of the baseline samples even if they are derived from a different, unsampled population. The GENECLASS program contains a 'simulation' option for assessing the probability of a multilocus genotype being derived from the allele frequencies of a population. A number of multilocus genotypes (in this case 10 000) are randomly generated, based on the estimates of allele frequencies of the specific population, and a frequency distribution of marginal probability values is generated. The marginal probability of a specific individual multilocus genotype is then compared to the distribution of marginal probabilities of randomly generated multilocus genotypes, and if the value is below a certain threshold probability level, say 5% or 1%, the individual is 'rejected' from the sample. We used this option for all samples, and for testing the usefulness of the approach we also assigned the two 'outgroup samples' (FLA and TJA) to the two baseline samples.

'Hybrids' between hatchery and wild trout are not immediately identified using assignment tests. We therefore used a hybrid index described by Campton & Utter (1985) for assessing if interbreeding took place between wild and hatchery trout. The hybrid index, I_H, was calculated as

$$I_H = 1 - \log(p_x)/[\log(p_x) + \log(p_y)]$$

where p_x denotes the likelihood of the multilocus genotype of an individual in population x and p_y similarly denotes the likelihood in population y (see Campton & Utter (1985) for details). If an allele is present in an individual but not in the sample for which the likelihood of the individual's genotype is calculated, the likelihood becomes zero. To avoid this problem alleles in individuals that were not present in a sample were arbitrarily given a frequency of 0.01. The hybrid index may range between 0 and 1 and, ideally, depending on the presence of sufficiently diagnostic loci, the score of individuals from the parental populations is close to either 0 or 1 whereas the scores of hybrids are intermediate. It is also worth noting that the hybrid index is closely related to the original assignment test developed by Paetkau *et al*. (1995); it can be regarded as a way of visualizing the relative assignment probabilities in an assignment test involving two samples. We first calculated hybrid indices of the individuals of the two baseline samples (HAT and WILD), based on the allele frequencies in these samples, and then calculated hybrid indices for the individuals in the suspected mixed samples of resident and sea trout, also based on the allele frequencies of the baseline samples.

Results

Single locus statistics

The total number of alleles at the microsatellite loci ranged from four (*Str* 60) to 15 (*SsHae*III*14.20*; Table 2). Only a few private alleles were observed, most of them in TJA and FLA and all at a frequency below 0.05. Three alleles were observed in the hatchery baseline sample (HAT) at relatively high frequencies that were not observed in the baseline sample from the wild population (WILD): *SsoSL* 417, 191 bp at a frequency of 0.09, *Ssa* 85, 118 bp (0.06) and *SsHae*III*14.20*, 330 bp (0.10). None of these alleles were observed among anadromous trout from the stocked section of the Karup River (SEA93, SEA93B and SEA98). However, all three alleles were observed among resident trout (RES93

Table 2 Summary of microsatellite data: observed number of alleles per locus and sample (number of private alleles in parentheses), outcome of tests for deviations from expected Hardy–Weinberg proportions, expected (H_E) and observed heterozygosity (H_O). Table-wide significance levels were applied, using the sequential Bonferroni technique (Rice 1989) (k = 63)

Microsatellite locus		HAT	WILD	RES93	RES98	SEA93	SEA98	SEA-B93	TJA	FLA
Str 15	No. of alleles	4 (0)	5 (0)	5 (0)	4 (0)	4 (0)	5 (0)	5 (0)	4 (0)	6 (2)
Total no.	H.-W. test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***
alleles: 7	H_E	0.667	0.710	0.690	0.447	0.698	0.676	0.777	0.722	0.788
	H_O	0.551	0.700	0.585	0.467	0.692	0.611	0.680	0.682	0.534
Str 60	No. of alleles	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)	3 (0)	4 (1)	3 (0)
Total no.	H.-W. test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
alleles: 4	H_E	0.376	0.538	0.515	0.556	0.618	0.540	0.522	0.477	0.507
	H_O	0.306	0.500	0.561	0.600	0.462	0.556	0.560	0.409	0.483
SsoSL 417	No. of alleles	9 (0)	7 (0)	9 (0)	7 (0)	8 (0)	10 (0)	8 (0)	10 (1)	12 (0)
Total no.	H.-W. test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**
alleles: 13	H_E	0.779	0.751	0.850	0.859	0.810	0.802	0.730	0.844	0.885
	H_O	0.714	0.740	0.756	0.800	0.923	0.833	0.760	0.886	0.845
SsoSL 438	No. of alleles	5 (0)	5 (0)	5 (0)	4 (0)	3 (0)	3 (0)	6 (0)	5 (0)	6 (1)
Total no.	H.-W. test	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***
alleles: 7	H_E	0.697	0.587	0.685	0.690	0.522	0.578	0.646	0.568	0.662
	H_O	0.510	0.720	0.683	0.600	0.462	0.611	0.720	0.523	0.483
Ssa 85	No. of alleles	5 (0)	6 (0)	6 (0)	6 (0)	5 (0)	6 (0)	6 (0)	6 (0)	4 (0)
Total no.	H.-W. test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
alleles: 7	H_E	0.647	0.752	0.692	0.740	0.765	0.784	0.700	0.761	0.688
	H_O	0.612	0.780	0.610	0.600	0.538	0.833	0.800	0.841	0.741
Ssa 197	No. of alleles	7 (0)	10 (1)	9 (0)	7 (0)	5 (0)	8 (0)	7 (0)	7 (0)	8 (0)
Total no.	H.-W. test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
alleles: 10	H_E	0.818	0.754	0.732	0.859	0.615	0.702	0.775	0.844	0.791
	H_O	0.939	0.780	0.634	0.733	0.385	0.722	0.840	0.886	0.845
SsHaeIII14.20	No. of alleles	9 (0)	8 (0)	9 (0)	5 (0)	7 (0)	7 (0)	7 (0)	12 (1)	13 (1)
Total no.	H.-W. test	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***
alleles: 15	H_E	0.765	0.830	0.771	0.747	0.845	0.826	0.817	0.827	0.762
	H_O	0.816	0.860	0.805	0.533	0.615	0.833	0.680	0.818	0.621

**Significant at the 1% level; ***significant at the 0.1% level; n.s., not significant.

Table 3 Mitochondrial DNA haplotype frequencies and nucleon diversity of the sampled populations. Haplotype nomenclature is according to Hansen & Loeschcke (1996) and Hansen *et al*. (1997)

mtDNA haplotype	HAT	WILD	RES93	RES98	SEA93	SEA98	SEA-B93	TJA	FLA
#2	0.41	0	0.24	0.07	0	0.06	0	0.22	0.05
#3	0	0	0.10	0	0	0	0	0.02	0
#4	0	0.50	0.24	0.40	0.77	0.56	0.80	0.06	0.37
#5	0.57	0.36	0.24	0.27	0.16	0.11	0.04	0.16	0.58
#6	0	0	0	0	0	0	0	0.04	0
#7	0.02	0	0	0	0	0	0	0.16	0
#8	0	0.14	0.17	0.20	0.08	0.28	0.16	0.06	0
#9	0	0	0	0	0	0	0	0.02	0
#10	0	0	0	0	0	0	0	0.24	0
#14	0	0	0	0.07	0	0	0	0.04	0
Nucleon diversity	0.52	0.41	0.81	0.77	0.41	0.62	0.34	0.85	0.54

and RES98). A total of five significant departures from Hardy–Weinberg equilibrium were observed (Table 2). Four of these departures, all associated with heterozygote deficiencies, were found in the FLA sample and could be due to a Wahlund effect as the fish were collected from two sites approximately 3 km apart in the river. Some of the other samples were small and the power of the tests may have been limited. However, testing for Hardy–Weinberg equilibrium after pooling all resident and anadromous samples, respectively, did not result in any significant departures.

We observed 10 mtDNA haplotypes (Table 3). They have all been observed previously in Danish trout populations. For restriction morph and haplotype nomenclature we refer to Hansen & Loeschcke (1996) and Hansen *et al*.

Table 4 Tests for genetic differentiation between pairs of samples. Above the diagonal, first line: exact tests for differences in microsatellite allele frequencies between samples. Above the diagonal, second line: pairwise F_{ST} values based on microsatellite loci. Below the diagonal: exact tests for differences in mtDNA haplotype frequencies between samples. Table-wide significance levels were applied, using the sequential Bonferroni technique (Rice 1989) (k = 36)

	HAT	WILD	RES93	RES98	SEA93	SEA98	SEA-B93	TJA	FLA
HAT		***	***	***	***	***	***	***	***
		0.060***	0.021*	0.059***	0.075***	0.061***	0.053***	0.025***	0.088***
WILD	***		***	***	n.s.	n.s.	n.s.	***	***
			0.019***	0.040***	0 n.s.	0.001 n.s.	0.006 n.s.	0.038***	0.043***
RES93	***	***		***	**	**	***	***	***
				0.049***	0.020 n.s.	0.014 n.s.	0.024*	0.021***	0.053***
RES98	***	n.s.	n.s.		***	**	***	***	***
					0.049*	0.027 n.s.	0.029*	0.044**	0.078***
SEA93	***	n.s.	n.s.	n.s.		n.s.	n.s.	***	***
						0 n.s.	0.010 n.s.	0.045**	0.037***
SEA98	***	n.s.	n.s.	n.s.	n.s.		n.s.	***	***
							0.007 n.s.	0.036***	0.042***
SEA-B93	***	n.s.	**	n.s.	n.s.	n.s.		***	***
								0.044***	0.048***
TJA	***	***	***	***	***	***	***		***
									0.037***
FLA	***	**	***	n.s.	*	***	***	***	

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s., not significant.

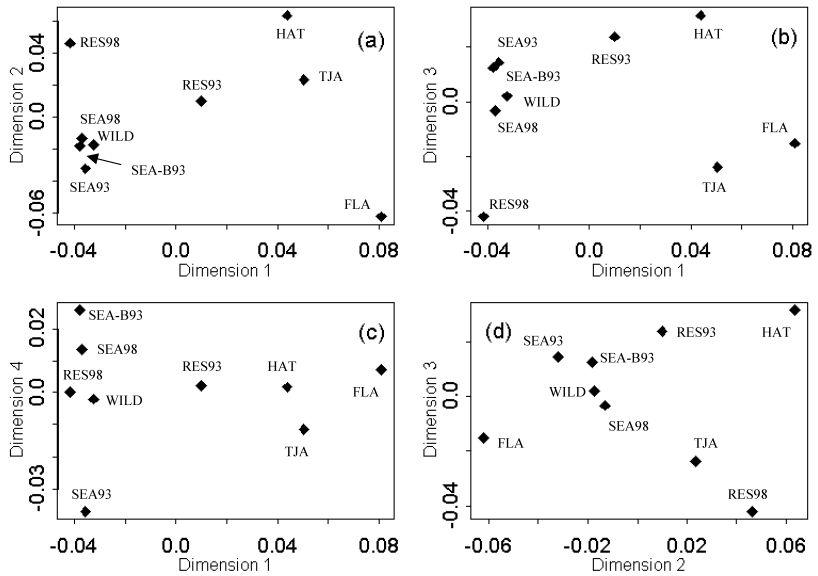

Fig. 2 Multidimensional scaling, based on seven microsatellite loci and using Nei *et al.*'s (1983) D_A distance. Plots of scaling components: (a) 1 (46.6% of variance) vs. 2 (32.4% of variance); (b) 1 vs. 3 (11.7% of variance); (c) 1 vs. 4 (6.3% of variance) (d) 2 vs. 3.

(1997). Some haplotypes were present at relatively high frequencies in the baseline sample from the Karup River (WILD; haplotypes 4 and 8) but not in hatchery sample (HAT), although haplotype 4 has previously been found at low frequency in a much more intensive screening of mtDNA variability among fish from this hatchery (Hansen *et al*. 1997). Haplotype 2 was observed at a high frequency in the hatchery sample (HAT) but was absent in the sample from the wild population (WILD). This haplotype was observed in only one individual among the sea trout from the stocked section of the Karup River, but was common in resident trout. Haplotypes 3 and 14 were observed at low frequencies in resident trout but not in the samples of sea trout. However, in a previous study by Hansen & Mensberg (1998) these haplotypes were also observed in other samples from the Karup River system.

Pattern of genetic differentiation among samples

Significant genetic differentiation was observed between the hatchery trout (HAT) and all other samples both at the microsatellite and mtDNA level (Table 4). Similarly, the two

outgroup samples, TJA and FLA, were significantly different from all other samples, except for the test for mtDNA differentiation between FLA and RES98, probably as a result of low power due to the small size of RES98 (N = 15). No significant differentiation was observed between the baseline wild sample from the Karup River (WILD) and the samples of sea trout from the stocked part of the river (SEA93, SEA98 and SEA-B93), whereas differentiation was significant between each of these samples and the resident trout (RES93 and RES98). Microsatellite differentiation was also significant between RES93 and RES98.

The pattern of differences and similarities among samples was visualized with a multidimensional scaling analysis applied to the matrix of D_A genetic distances (Fig. 2). A plot of dimension 1 (which explained 46.6% of the variance) vs. dimension 2 (32.4% of the variance, Fig. 2a) indicated that the Flads and Tjærbæk river samples (FLA and TJA) and the sample of hatchery trout (HAT) were the most divergent along dimension 1 followed by the sample of resident trout from 1993 (RES93). These same samples, as well as that of resident trout from 1998 (RES98), were also the most divergent along dimension 2 (Fig. 2a). Conversely, this plot suggested a high degree of similarity between the baseline sample of Karup River trout (WILD) and the three samples of sea trout caught above or below the barrier (SEA93, SEA-B93, SEA98). A plot of dimensions 1 vs. 3 (11.7% of variance, Fig. 2b) suggested again a high degree of similarity along dimension 3 among the baseline sample of wild Karup River trout and the three samples of sea trout from above or below the barrier. The sample of resident trout from 1998 (RES98), the sample from the Tjærbæk river (TJA), and to a lesser degree the samples from the Flads river (FLA) and the hatchery (HAT) differed most from the rest along dimension 3 (Fig. 2b). Differences among the baseline sample of wild Karup River trout (WILD) and the three samples of sea trout became apparent only along dimension 4 which explained only 6.3% of the variance (Fig. 2c). Finally, a plot of dimensions 2 and 3 (Fig. 2d) again suggested a higher degree of genetic similarity of the wild Karup River trout (WILD) with the SEA93, SEA-B93 and SEA98 samples, than with the sample from the hatchery strain used to stock the river (HAT), the two samples of resident trout, or the two outgroup samples from the Flads and Tjærbæk rivers, respectively (FLA, TJA).

Assignment tests and hybrid indices

The assignment tests (Table 5) showed that nearly all individuals from the two baseline samples, HAT and WILD, were assigned correctly and a high proportion were 'rejected' from the sample that they were not derived from. Between 85% and 100% (93% in total) of sea trout from the stocked river section were assigned to the baseline sample of wild trout (WILD) and the majority of individuals were 'rejected' (at the 5% level) from the hatchery sample (HAT). In contrast, approximately equal proportions of resident trout from 1993 (RES93) were assigned to the hatchery (HAT) and the wild baseline sample (WILD), and some individuals from the small RES98 sample were also assigned to HAT. In total, 54% of all resident trout (RES93 and RES98 pooled) were assigned to WILD and 46% were assigned to HAT.

Only a few individuals from HAT and WILD were rejected from both samples (10% at the 5% level and 8% at the 1% level, using the simulation option in GENECLASS;

Table 5 Results of assignment tests, using the Bayesian approach included in the program GENECLASS 1.0 (Cornuet *et al*. 1999). 'Rejected' denotes the proportion of individuals with multilocus genotypes that were among the 5% or 1% most unlikely genotypes in one of the two baseline samples

Sample	N	HAT	WILD	Rejected from both samples
HAT	49			
assigned to		0.92	0.08	
rejected (5% criterion)		0.10	0.63	0.10
rejected (1% criterion)		0.08	0.49	0.08
WILD	50			
assigned to		0.06	0.94	
rejected (5% criterion)		0.76	0.10	0.10
rejected (1% criterion)		0.48	0.08	0.08
SEA 93	13			
assigned to		0.15	0.85	
rejected (5% criterion)		0.54	0.23	0.23
rejected (1% criterion)		0.54	0.15	0.15
SEA 98	18			
assigned to		0	1.0	
rejected (5% criterion)		0.72	0.17	0.17
rejected (1% criterion)		0.33	0.06	0.06
SEA-B93	25			
assigned to		0.08	0.92	
rejected (5% criterion)		0.80	0.16	0.16
rejected (1% criterion)		0.36	0.04	0.04
RES93	41			
assigned to		0.54	0.46	
rejected (5% criterion)		0.44	0.29	0.12
rejected (1% criterion)		0.22	0.22	0.02
RES98	15			
assigned to		0.27	0.73	
rejected (5% criterion)		0.60	0.40	0.13
rejected (1% criterion)		0.47	0.13	0
TJA	44			
assigned to		0.55	0.45	
rejected (5% criterion)		0.64	0.64	0.45
rejected (1% criterion)		0.41	0.41	0.16
FLA	58			
assigned to		0.36	0.64	
rejected (5% criterion)		0.90	0.79	0.74
rejected (1% criterion)		0.76	0.66	0.57

Table 5). Their multilocus genotypes had a low probability of being derived from the allelic composition of any of these two populations and they could, in principle, belong to other, unsampled populations. The proportions of individuals from RES93, RES98, SEA93, SEA98 and SEA-B93 that were rejected from both WILD and HAT were of the same magnitude or slightly higher, compared to the individuals from the baseline samples. In contrast, a much larger proportion of the individuals from the two outgroup populations, TJA and FLA, was rejected from both WILD and HAT (Table 5).

For calculating hybrid indices a combination of the three most polymorphic loci, *SsoSL* 417, *Ssa* 197 and *SsHae*III*14.20*, provided the best discrimination between the baseline samples of hatchery (HAT) and wild Karup River (WILD) trout. The distribution of hybrid indices for individuals from the two baseline samples pooled is shown in Fig. 3a. Values close to 0 should indicate a fish with typical wild Karup River (WILD) allelic composition. Conversely, individuals with values close to 1 should indicate a fish with typical hatchery (HAT) allelic composition. In reality, discriminatory power was weak due to the large number of alleles shared between both populations and there was overlap in hybrid indices of individuals from the two samples. Nevertheless, a bimodal distribution of the indices was also apparent. The hybrid indices in sea trout from the stocked section of the river were mainly below 0.5 (Fig. 3b). This was expected, as the majority of individuals were assigned to WILD (Table 5). Approximately equal proportions of resident trout were assigned to HAT and WILD. However, contrary to Fig. 3a an excess of intermediate hybrid indices was observed (Fig. 3c). Even though the two distributions of hybrid indices (Fig. 3a vs. 3c) were not significantly different when a Kolmogorov–Smirnov two-sample test was applied the result was very close to significance ($D = 0.221$; $D = 0.227$ would be significant at the 5% level and $D = 0.205$ at the 10% level).

Finally, we calculated mtDNA haplotype frequencies of the resident trout (RES93 and RES98 pooled) that were assigned to HAT and WILD, respectively. Two individuals (7%) assigned to WILD exhibited the hatchery-specific haplotype 2, whereas 10 individuals (38%) assigned to HAT exhibited haplotypes 4 and 8 that were specific to WILD (Table 6).

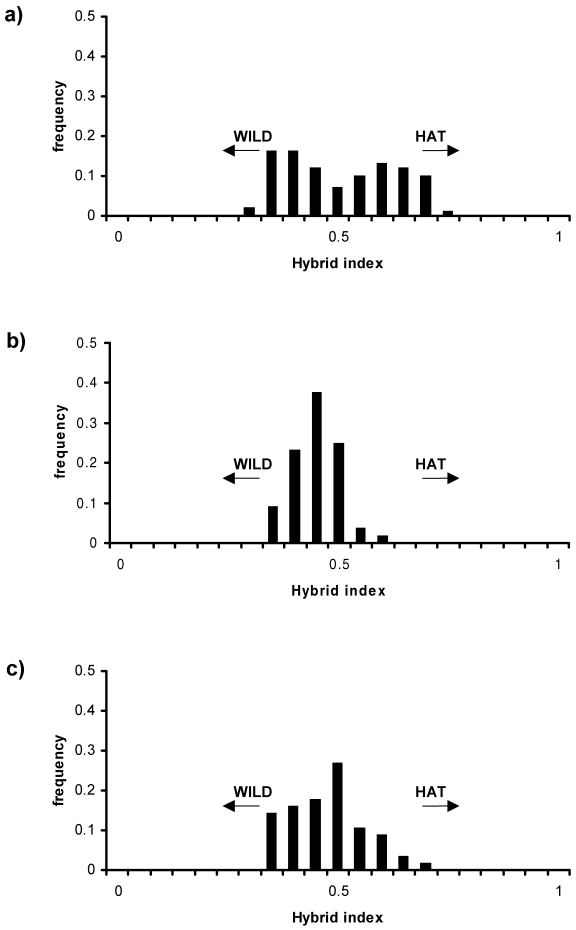

Fig. 3 Frequency distributions of hybrid indices, calculated according to Campton & Utter (1985) and based on the loci *SsoSL* 417, *Ssa* 197 and *SsHae*III*14.20*. (a) Hybrid indices of individuals from the baseline samples (HAT and WILD pooled). (b) Hybrid indices of sea trout from the stocked section of the Karup River (SEA93, SEA98 and SEA-B93 pooled). (c) Hybrid indices of resident trout from the stocked section of the Karup River (RES93 and RES98 pooled).

mtDNA haplotype	WILD	RES93 and RES98 assigned to WILD	HAT	RES93 and RES98 assigned to HAT
#2	0	0.07 (2)	0.41 (20)	0.35 (9)
#3	0	0.10 (3)	0	0.04 (1)
#4	0.50 (25)	0.33 (10)	0	0.23 (6)
#5	0.36 (18)	0.27 (8)	0.57 (28)	0.23 (6)
#7	0	0	0.02 (1)	0
#8	0.14 (7)	0.20 (6)	0	0.15 (4)
#14	0	0.03 (1)	0	0
n	50	30	49	26

Table 6 mtDNA haplotype frequencies in individuals of resident trout (pooled samples RES93 and RES98) assigned to WILD and HAT, respectively. For comparison, the haplotype frequencies of WILD and HAT are also listed

Discussion

Proportions of wild and domesticated fish among resident and sea trout

The multidimensional scaling analysis (MDSA) plots (Fig. 2) clearly demonstrated close genetic relationships between wild Karup River trout (WILD) and the samples of sea trout from the stocked section of the Karup River (SEA93, SEA98 and SEA-B93). Conversely, resident trout (RES93 and RES98) sampled from the same sections of the river as the samples of sea trout were genetically divergent, and the same applied to the hatchery strain (HAT) and the two outgroup samples TJA and FLA. The assignment tests (Table 5) provided further information regarding the composition of the samples of resident and sea trout. Despite only modest differentiation between HAT and WILD ($F_{ST} = 0.06$) we were able to assign individuals to their population of origin with high success (> 90%; Table 5) using seven microsatellite loci. Sea trout collected in the stocked section of the Karup river were nearly all assigned to WILD, and we conclude that there were few, if any, hatchery trout among the sea trout. In contrast, the presence of some hatchery-specific microsatellite alleles and mtDNA haplotype 2 among resident trout (Table 3) suggested that hatchery trout and/or their descendants were included in the samples of resident trout. The contribution of hatchery trout to the pool of resident fish was estimated to be approximately 50%, but it seemed to be decreasing from 1993 to 1998 (Table 5). It is in principle possible for some or all of the sampled resident trout to belong to an unsampled population instead of either the HAT or WILD populations. This poses a potential problem for the interpretation of the assignment tests. We tested for this possibility by assigning individuals from two outgroup samples of known origin (TJA and FLA) to the same two baseline populations. As with the test using resident trout, approximately equal proportions of each of the TJA and FLA samples were assigned to HAT and WILD. However, the tests for assessing if a multilocus genotype was likely to occur, given the allele frequencies of the hatchery and the wild trout (HAT and WILD, respectively), showed that a large proportion of the multilocus genotypes in TJA and FLA were 'rejected' from both baseline samples (Table 5). This was not observed in the tests using true resident samples, where the proportions 'rejected' from either baseline component were small and did not differ from those observed in the tests using sea trout. We therefore conclude that the samples of resident trout consisted of approximately equal proportions of Karup River and hatchery trout.

Genetic interactions between wild and domesticated trout

Assignment tests alone did not provide evidence for interbreeding between wild and hatchery trout. The presence of resident, hatchery trout could be explained by the fact that stocking in the past had included older resident trout. The hybrid indices of resident trout indicated, however, an excess of intermediate values (Fig. 3c) where in fact a bimodal distribution would have been expected in the absence of interbreeding (Fig. 3a). Further evidence for interbreeding was provided by the mtDNA haplotype frequencies among individuals assigned to the hatchery (HAT) and the indigenous (WILD) populations, respectively (Table 6). It is unlikely that the 38% of haplotypes specific to the indigenous Karup River trout observed in trout assigned (using only microsatellites) to the hatchery population (HAT) could be the result of misassignment. The proportion of hatchery-specific haplotypes in individuals assigned to WILD was much lower (7%). This suggests that the major part of interbreeding between wild and hatchery fish involved Karup River female trout and hatchery male trout. In order to assess this we made the following approximate assumptions: First, we assumed that all 'hybrids' were F1. Second, we assumed that the proportions of F1 'hybrids' among trout assigned to HAT and WILD were equal. This is not unreasonable, as the assignment tests showed that approximately equal proportions of all resident trout were assigned to HAT and WILD, respectively. Also, equal proportions of F1 'hybrids' (exhibiting intermediate genotypes) would be expected to be assigned to HAT and WILD. By dividing the 38% of haplotypes specific to WILD but observed in individuals assigned to HAT by the frequency of these haplotypes in WILD (64%) we obtain an approximate estimate of the maternal contribution to interbreeding by wild trout of 59%. A similar estimate based on hatchery (HAT) haplotypes observed in trout assigned to WILD yields a result of 0.07/0.41 = 17%, indicating a larger contribution to interbreeding by male hatchery trout than by female hatchery trout. It should be stressed, however, that it is a rough estimate and sampling variance is not taken into account.

Even though the proportion of hatchery trout among the samples of sea trout appeared very low, it is still likely that a few 'hybrids' were included. In particular, one sea trout exhibited the hatchery-specific haplotype 2 (Table 3). This individual was assigned to WILD, but the marginal probability of the individual belonging to the hatchery population (HAT) was almost the same, so it is possible for this individual to represent a hatchery female–wild male 'hybrid'.

It is surprising that there were very few traces of hatchery

trout among sea trout from the stocked section of the Karup River, whereas there was a considerable component of hatchery trout among resident trout from the same section. In addition, there was evidence of interbreeding between wild and hatchery trout within this group. Hindar *et al*. (1991b) demonstrated negligible genetic differentiation between coexisting resident and anadromous trout, probably reflecting extensive interbreeding between the two forms, and we assume that resident and sea trout also interbreed freely in the Karup River. A simple explanation of the results could be that all stocked hatchery trout became resident. Even though this is possible, there is no evidence of reduced smoltification among trout from the Hårkær Hatchery strain. It is in fact one of the most important suppliers of smolts for stocking elsewhere in Denmark, although it should be noted that smoltification is influenced by environmental factors (Elliott 1994) and the proportion of stocked hatchery trout that smoltify in the Karup River may not be the same as in the hatchery. It is also possible that stocked trout that become sea trout exhibit poorer homing compared to indigenous fish and stray to other rivers (e.g. Stabell 1984). However, we find it unlikely that this alone should result in a return rate close to zero, as suggested by the present results. It appears more likely that stocked hatchery trout perform poorer as sea trout than as resident trout, probably as a result of the differences in complexity of the two kinds of life cycles. The life history of sea trout is highly complex and various factors, such as timing of smoltification and migratory behaviour in the sea, may contain genetic components and reflect specific adaptations (e.g. Svärdson & Fagerström 1982). In contrast, stocked hatchery trout that become resident experience a comparatively more stable environment and may exhibit higher survival rates.

The sex ratio in brown trout has been found to differ among resident and anadromous trout with approximately 75% of all sea trout being females, whereas in resident trout the proportion of males is high, ranging from approximately 75% to 100% (Jonsson 1982; Rasmussen 1986). It was not possible to determine the sex of all resident trout, but among 126 sea trout from the Karup River, including the samples of this study, 97 were females and 29 males, which is in accordance with the observations mentioned above. Consequently, even though our results concerning male-biased gene flow from hatchery trout to wild trout were not conclusive it would not be unexpected if interbreeding involved primarily male hatchery trout and female wild trout. We hypothesize the following: a proportion of stocked hatchery trout would smoltify and become sea trout, but only few would return to spawn, probably due to low survival. The majority of female hatchery trout would be included in this group. The other proportion of stocked hatchery trout, the majority of which are males, would become resident and exhibit higher survival than hatchery sea trout. Mature hatchery males, if able to reproduce, would in most cases mate with wild female trout, most of which are sea trout. Differences in survival between anadromous and resident hatchery trout would thereby result in male-biased gene flow from hatchery trout to wild trout, as indicated by our results.

Interbreeding between domesticated and wild salmonids has been demonstrated in several studies (e.g. Taggart & Ferguson 1986; Largiadèr & Scholl 1996). Relatively fewer studies have attempted to assess if the genetic contribution by domesticated fish was permanent. However, studies by Chilcote *et al*. (1986), Skaala *et al*. (1996) and Poteaux *et al*. (1998) showed that natural selection was indeed acting against domesticated fish and 'hybrids'. In the case of the Karup River a previous study, employing mtDNA analysis, showed that after stocking with hatchery trout had stopped in the main part of the river, a genetic contribution by the stocked trout could hardly be detected (Hansen *et al*. 1995). However, it was not known if interbreeding between wild and hatchery trout had in fact taken place or if the hatchery trout had simply been unable to survive and reproduce. The present results suggest that interbreeding did take place. However, the genetic contribution by hatchery trout was later removed, presumably by natural selection, although disproportional mortality of wild and hatchery trout due to angling could also be an important factor (Garcia-Marin *et al*. 1998).

Only a few studies have focused on the use of microsatellite markers for estimating stocking impact and introgression. Brunner *et al*. (1998) used sharing of alleles between stocked and nonstocked Arctic charr (*Salvelinus alpinus* L.) populations as indicators of introgression. Two other studies have attempted to quantify 'maximum introgression rate' on the basis of alleles shared between hatchery strains and stocked populations (Poteaux *et al*. 1999) and alleles present in hatchery strains but absent in stocked populations (Hansen *et al*. 2000). The latter two approaches do not provide evidence that introgression has taken place, but instead the maximum rate of introgression compatible with the data is calculated. The present study shows that if baseline samples are available from the hatchery strain and from the stocked population, either prior to stocking or in a state where it can be considered unaffected by stocking, it is possible to estimate the genetic contribution by hatchery trout and detect interbreeding, even if genetic differentiation is small. Whereas the hybrid indices did indicate interbreeding between wild and hatchery trout (Fig. 3), we also found that resolution was low. We favour the approach of combining assignment tests based on microsatellites with information on mtDNA haplotypes of the assigned individuals.

This procedure allowed us to detect interbreeding and indicated that gene flow from hatchery to wild trout was male biased.

In a wider conservation genetics context the present study demonstrates the applicability of microsatellite analysis and assignment tests for monitoring gene flow from captive or translocated populations to wild indigenous populations. Such problems are not restricted to fishes, but occur also in other organisms, such as plants (e.g. Ellstrand 1992) and game animals (e.g. Thulin *et al*. 1997).

Conclusions

In conclusion, we found that assignment tests were very useful for distinguishing wild and domesticated trout. A significant genetic contribution by hatchery trout was found only in samples of resident trout and there were indications that male hatchery trout contributed more to interbreeding than hatchery females. We suggest that this is due to differential survival between domesticated trout exhibiting resident and anadromous life histories, possibly combined with uneven sex ratios within the two life-history forms. Our results suggest that even in the case of modest differentiation at neutral loci domesticated trout may still perform significantly worse than local wild populations. This could result from both the existence of local adaptations in general and the effects of domestication in hatchery trout.

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