

# Genetic monitoring of supportive breeding in brown trout (*Salmo trutta* L.), using microsatellite DNA markers

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**Abstract:** Stocking with offspring of local wild fish, so-called supportive breeding, is often advocated as an alternative to stocking domesticated fish. However, it is important to ensure that supportive breeding does not result in inbreeding and loss of genetic variability. We analysed eight microsatellite loci in samples of wild and hatchery-reared brown trout (*Salmo trutta*) from three populations subject to supportive breeding. For calibrating statistical procedures, we included two test samples of reared offspring for which the precise number of parent fish was known and a sample from a further wild reference population. Three different statistical procedures were used to detect population bottlenecks and loss of variability: (i) a randomization test for comparing allelic diversity between samples; (ii) estimates of effective number of breeders from gametic-phase disequilibrium; and (iii) a test for assessing population bottlenecks based on detecting deviations from mutation-drift equilibrium. All three procedures were useful but they also exhibited different strengths and limitations, with the test for population bottlenecks probably being the single most useful procedure for routine monitoring. In two populations subject to supportive breeding, there were strong indications of reduced effective population sizes, and significant genetic differentiation was observed between different samples from the same population.

**Résumé :** Le repeuplement par la progéniture des poissons sauvages indigènes, c'est-à-dire le produit d'une sélection de soutien, est souvent préconisé pour remplacer le repeuplement par des poissons domestiqués. Il est toutefois important de vérifier que cette sélection de soutien ne se traduira pas par l'endogamie et par une perte de variabilité génétique. Nous avons analysé huit locus microsatellites chez des truites brunes (*Salmo trutta*) sauvages et élevées en écloserie provenant de trois populations soumises à la sélection de soutien. Pour étalonner nos méthodes statistiques, nous avons inclus deux échantillons expérimentaux de poissons issus d'élevage pour lesquels on connaissait précisément le nombre de géniteurs, et un échantillon provenant d'une autre population sauvage de référence. Trois méthodes statistiques différentes ont été employées pour détecter les goulots d'étranglement démographiques et la perte de variabilité : (i) un test de randomisation pour comparer la diversité allélique d'un échantillon à l'autre; (ii) des estimations du nombre effectif de géniteurs à partir du déséquilibre de la phase gamétique; et (iii) un test pour évaluer les goulots d'étranglement démographiques basé sur la détection des écarts par rapport à l'équilibre mutation-dérive. Les trois méthodes se sont révélées utiles, mais présentaient aussi des forces et des limitations diverses; c'est le test des goulots d'étranglement qui était probablement la méthode la plus utile pour une surveillance régulière. Dans deux populations soumises à la sélection de soutien, on notait de fortes indications d'une réduction de la taille effective de la population, et une différenciation génétique significative a été observée entre différents échantillons de la même population.

[Traduit par la Rédaction]

## Introduction

Human activities are rapidly altering the living conditions of many fish species as a result of overfishing, pollution, and alteration and degradation of habitats. In some cases, this may lead to the entire loss of populations, whereas in other

cases, populations may, in the long term, be able to adapt to the changes in environmental conditions. However, the presence of sufficient genetic variability is a prerequisite to being able to respond to altered selection regimes (Hedrick and Miller 1992).

Stocking non-native domesticated fish into wild fish populations compromises the genetic variability of wild populations, as locally adapted populations exhibiting high levels of genetic variation may be swamped by nonadapted genetically depauperate domesticated fish (Hindar et al. 1991). This has particularly been a concern in the case of salmonid fishes and, as a consequence, the use of local wild spawners for producing stocking material has been advocated (e.g., Waples 1991a; Hansen and Loeschcke 1994), a procedure usually referred to as "supportive breeding." Even though supportive breeding may be a useful management and conservation tool, it is not without its limitations. Ryman and Laikre (1991) showed that the total effective population size

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may decrease considerably if the effective number of breeders (usually denoted by  $N_b$ ) used for artificial propagation is small and a large proportion of all offspring is made up by the stocked individuals. Waples and Do (1994) performed computer simulations to study the effects of supportive breeding on inbreeding and obtained results qualitatively similar to those of Ryman and Laikre (1991). In particular, they demonstrated that, if a small population is artificially increased by supportive breeding, the use of a small number of breeders may result in an "inbreeding crash," if supportive breeding later ceases and natural reproduction has not increased. These results stress the importance of maintaining sufficiently large effective population sizes in supportive breeding and, consequently, genetic monitoring of supportive-breeding activities is recommended. However, so far only a few empirical studies have been undertaken that address effective population sizes in supportive breeding (e.g., Hedrick et al. 1995), and there are few examples of genetic markers being used for this purpose (Bartley et al. 1992).

Allozymes have proved useful for detecting loss of variability in hatchery strains (e.g., Ryman and Ståhl 1980). Nevertheless, the low variability at allozyme loci in most salmonid species, involving primarily diallelic polymorphism, reduces their sensitivity. In contrast, some nuclear DNA markers, such as mini- and micro-satellites, exhibit high levels of polymorphism and many rare alleles. Therefore, these kinds of markers are expected to be particularly useful for detecting reduced effective population sizes and loss of variability, and they have been applied successfully in studies aimed at assessing variability in hatchery-reared versus wild populations of salmonid fishes (e.g., Stone et al. 1997; Primmer et al. 1999; Hansen et al. 2001).

Here we present the results of a study of brown trout (*Salmo trutta* L.), which is subject to supportive breeding in several rivers in Denmark. Samples were screened for variation at eight microsatellite loci. We estimated the genetic relationship among populations and used three different approaches to assess whether or not bottlenecks and (or) loss of variability occurred during artificial propagation. First, we used a randomisation test to compare the variability of hatchery-reared trout with that of the wild source populations (see Nielsen et al. 1999). Second, we estimated the effective number of breeders per spawning season ( $N_b$ ) in hatchery-reared trout based on gametic-phase disequilibria (Hill 1981; Waples 1991b; Bartley et al. 1992). Third, we used the test developed by Cornuet and Luikart (1996) to detect population bottlenecks. We compare the three methods and discuss the management implications of the results.

## Materials and methods

### Sampled populations

We concentrated on three different rivers in which the trout populations are subject to supportive breeding, viz., the Skjern, Karup, and Esrum rivers (see Fig. 1 for approximate localities and Table 1 for basic information about samples). All populations consist of a mixture of resident and anadromous trout. The Skjern River trout population has presumably been small for the past two decades, with ca. 10–20 sea trout caught per year by anglers. Supportive breeding in this population is based on maintaining a "gene bank", consisting of 25–50 adult males and 25–50 adult females, in a hatchery. Each year, this gene bank is supplemented by wild

spawners caught in the Skjern River. We obtained samples of offspring from the hatchery in 2 different years, but did not obtain samples of wild spawners from the river. At the present time, the sea trout run in the Karup River is large (several thousand spawners per year) but, from the 1970s to the early 1980s, the population declined drastically, owing to overfishing and poor environmental conditions, and the population may have experienced a genetic bottleneck. Supportive breeding in this population is based on 100–200 wild spawners that are all caught in the river each year by electrofishing, i.e., no gene bank is maintained. We obtained two samples of wild spawners from this river (in 1993 and 1996) and a sample from the hatchery in which the stocking material is reared. The Esrum River trout population is considered to be small. The river is an outlet of a lake and, in recent years, toxic algae from this lake have repeatedly killed juvenile trout in the river. Supportive breeding in this population is based on ca. 50 wild spawners (ca. 25 males and 25 females) that are caught each year by electrofishing. We obtained samples of wild spawners and sampled two consecutive age-classes of offspring from the hatchery. We included a further reference sample from a population that is not subject to supportive breeding or stocking, viz., the population of the Tjærbæk River. Finally, for evaluating methods for assessing effective numbers of breeders from gametic-phase disequilibria, we included two samples of offspring from sets of experimental crosses that involved trout from commercial-hatchery strains, the Lysbro and Dront hatchery strains. The samples LYS and DRO represented offspring from eight and five full-sib families, respectively.

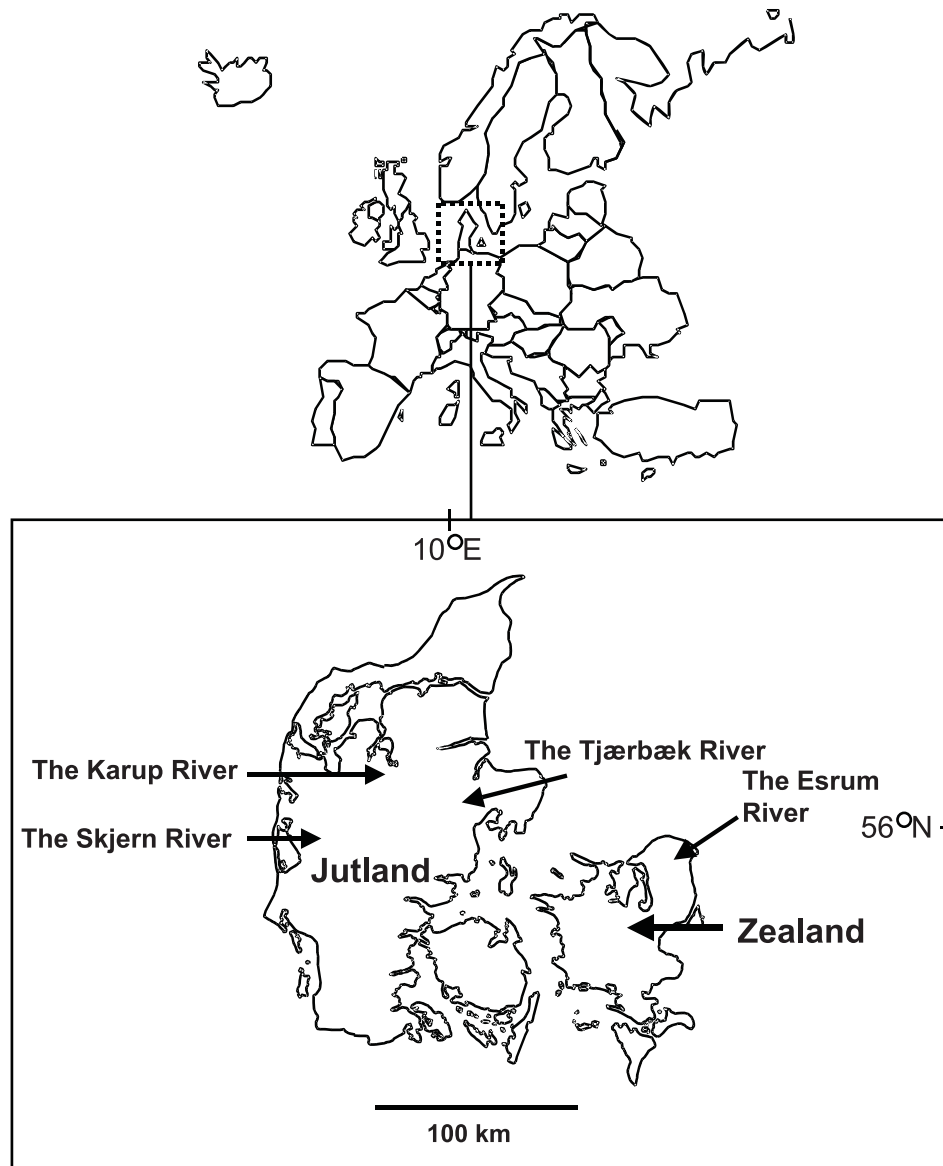
### Microsatellite analysis

DNA was extracted from muscle or adipose fin tissue stored in ethanol, using the Chelex – proteinase K extraction protocol of Estoup et al. (1996) or the phenol–chloroform extraction protocol of Taggart et al. (1992). One tetranucleotide and seven dinucleotide microsatellite loci were analysed: Str 15, Str 60, and Str 73 (Estoup et al. 1993); Ssa 85 and Ssa 197 (the tetranucleotide microsatellite) (O'Reilly et al. 1996); SsoSL 417 (Slettan et al. 1995) and SsoSL 438 (Slettan et al. 1996); and SsHaeIII.14.20 (J.L. Goodier, Department of Biochemistry, Memorial University of Newfoundland, St. John's, NF A1B 3X9, Canada, unpublished data; 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.

### Statistical treatment

Departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium or, rather, gametic-phase disequilibria, between pairs of loci were tested by exact tests using GENEPOP 3.1d (Raymond and Rousset 1995). Genetic differentiation between pairs of samples was analysed by calculating pairwise  $F_{ST}$  values and testing their significance by permuting individuals between samples using the program ARLEQUIN 1.1 (Schneider et al. 1997). The genetic relationship among samples was further analysed by multi-dimensional scaling analysis (MDSA) of a matrix of Cavalli-Sforza and Edwards' (1967) genetic distances between populations. MDSA was performed using the program VISTA 5.6.3 (Young 1996).

Reduced genetic variability (number of alleles) in hatchery-reared offspring compared with wild spawners from the same river was tested by a randomisation test (Manly 1997). The specific test used is described in more detail in Nielsen et al. (1999). Briefly, for each locus, it was assumed that the allele frequencies of the sample of wild spawners represented the true allele frequencies of the population. Samples equal in size to the sample of reared offspring were then drawn at random, and this procedure was repeated 1000 times. A frequency distribution of the number of alleles in the generated samples was constructed, and the probability of observing a number of alleles equal to or less than the number observed in the actual sample of offspring could be assessed. We tested for an

**Fig. 1.** Map showing the approximate locations of the sampling sites in Denmark.**Table 1.** Details of samples.

Sample locality	Sample abbreviation used	Status of population <sup>a</sup>	Year sampled	Sample size
Tjærbæk River	TJA	Wild, not stocked	1994	48
Karup River	KAR93	Wild spawners	1993	50
	KAR96	Wild spawners	1996	77
	KARSUP	Supportive breeding	1997	93
Skjern River	SKJSUP95	Supportive breeding	1995	60
	SKJSUP96	Supportive breeding	1996	60
Esrum River	ESR	Wild spawners	1996	54
	ESRSUP95	Supportive breeding	1995	59
	ESRSUP96	Supportive breeding	1996	79
Lysbro Hatchery	LYS	Offspring of 16 parent fish	1997	100
Dront Hatchery	DRO	Offspring of 10 parent fish	1997	100

<sup>a</sup>Wild spawners, trout reared for supportive breeding (supportive breeding), or wild population not subject to stocking (wild, not stocked).

overall reduction in genetic variability by combining probabilities from individual loci using Fisher's method.

Nb was estimated from gametic-phase disequilibria between loci (Hill 1981; Waples 1991b; Bartley et al. 1992). Random genetic drift tends to create gametic-phase disequilibria even between unlinked loci, and this can be used to estimate effective population size. In fact, if loci are unlinked, the major part of the gametic-phase disequilibrium will be generated in the present cohort (Waples 1991b), although Pudovkin et al. (1996) also pointed out that there may be some retention of disequilibria from previous generations. The average correlation between alleles at a pair of loci,  $r$ , was estimated using the program Linkdos (Garnier-Pere and Dillmann 1992). Nb could then be estimated as:

$$Nb = 1 \times (3(R^2 - (1 \times S^{-1})))^{-1}$$

where  $R^2$  denotes the arithmetic mean of squared correlations between alleles,  $r$ , at all pairwise combinations of loci and  $S$  denotes the harmonic mean of sample sizes. Ninety-five percent confidence intervals (CIs) were obtained by replacing  $R^2$  with CIs for  $R^2$  in the above equation. For further details, see Bartley et al. (1992). Waples (1991b) pointed out that this method for estimating Nb may only be valid for diallelic loci. In the case of multiallelic loci, one way to circumvent the problem would be to pool alleles. We therefore estimated Nb both from multiallelic-correlation values and by pooling all except the most common allele at each locus in one single composite allele.

Finally, we used the test procedures and the program Bottleneck 1.2 developed by Cornuet and Luikart (1996) to detect population bottlenecks. The bottleneck test is based on the fact that a population bottleneck reduces both the number of alleles and the *expected* heterozygosity ( $H_e$ ). However, the number of alleles is reduced at a faster rate than the  $H_e$ . Consequently, for some time after the bottleneck has occurred,  $H_e$  is higher than expected, given the alleles present at the loci, i.e., the population is not at mutation-drift equilibrium. Based on the alleles actually observed in the sample, the program estimates the *equilibrium* heterozygosity ( $H_{eq}$ ). We assumed an infinite allele mutation (IAM) model, a stepwise mutation (SM) model, or a two phase mutation (TPM) model. This last model, which is presently considered the most realistic for microsatellite loci (Estoup and Cornuet 1999), assumes that most mutations occur as stepwise mutations, but that a smaller fraction (5–10%) involves mutational changes in several repeat units. The estimated  $H_{eq}$  for each locus is then compared with the observed  $H_e$ . Several tests can be used to assess if a significantly high number of loci in a sample exhibits higher  $H_e$  than  $H_{eq}$ . As a relatively small number of loci (eight) were studied, we followed the recommendation of the authors and report the results obtained by a Wilcoxon's signed-ranks test. In all cases of multiple tests, significance levels were adjusted using the sequential Bonferroni technique, as recommended by Rice (1989).

## Results

The eight microsatellite loci showed medium to high levels of polymorphism, with numbers of alleles ranging from 5 to 15 (Table 2). In 15 of a total of 88 tests, significant deviations from HWE were detected (Table 2). Not surprisingly, in the test sample DRO, which represents the offspring of only 10 parent fish, five of eight loci deviated significantly from HWE, which represents a strong violation of the Hardy-Weinberg assumption of infinite population size. In the test sample LYS, which represents the offspring of 16 parent fish, only one significant deviation from HWE was observed. Four significant departures from HWE were observed in the sample of hatchery-reared trout from the Karup River (KARSUP),

and they were all associated with a deficiency of heterozygotes. Gametic-phase disequilibrium between pairs of loci was observed in only 4 of a total of 224 tests (excluding the samples LYS and DRO), and these involved different pairs of loci (data not shown). Overall, there were no indications of linkage of loci. Significant genetic differentiation was observed between all pairs of samples (excluding the samples LYS and DRO), except between the two samples of wild trout taken in the Karup River in 1993 and 1996 (KAR93 and KAR96; see Table 3). However, even though  $F_{ST}$  values between the sample of artificially propagated trout from the Karup River (KARSUP) and the two samples of wild trout (KAR93 and KAR96) were significant, they were also lower than  $F_{ST}$  values between other populations. This trend was also apparent in the MDSA of the matrix of pairwise Cavalli-Sforza and Edwards' (1967) distances (Fig. 2). A plot of dimension 1 (which explains 54% of the variance) versus dimension 2 (which explains 18% of the variance) showed that KAR93, KAR96, and KARSUP grouped closely together. In contrast, both samples of reared trout from the Skjern River (SKJSUP95 and SKJSUP96) were separated along dimensions 1 and 2, and strong divergence was also apparent between the sample of wild trout from the Esum River (ESR) and the two samples of reared trout from this same river (ESRSUP95 and ESRSUP96). The same patterns were observed in plots of dimension 1 versus dimension 3 (which explains 14% of the variance) and dimension 2 versus dimension 3 (not shown).

The tests for differences in number of alleles between wild spawners and propagated trout from the same river could only be performed in the case of the Karup and Esum river populations, as we did not have samples of wild trout from the Skjern River. In the case of the Karup River, we pooled the two samples of wild trout taken in 1993 and 1996 and based the tests on the allele frequencies of the pooled sample. There was no evidence of loss of variability due to supportive breeding in Karup River trout. In fact, at four of the eight loci, there were more alleles in the KARSUP sample than in the pooled sample of wild trout (Table 4). In Esum River trout, there were indications of reduced variability at three loci in both the ESRSUP95 and ESRSUP96 samples and, when probabilities from different loci were combined using Fisher's method, the results were highly significant (Table 4).

Estimates of Nb from linkage disequilibria were performed only for samples of hatchery-reared trout, including the two test samples LYS and DRO (Table 5). In the samples of wild spawners, it was obvious that several age-classes were present and, consequently, it was not possible to estimate Nb. In several cases, it was not possible to separate genetic drift (and, hence, gametic-phase disequilibria and Nb estimates) from sampling error. In accordance with Bartley et al. (1992) and Luikart and Cornuet (1999), these results were arbitrarily denoted by  $\infty$ . Estimates of Nb based on "multiallelic" correlations between pairs of loci were nearly always higher than those based on "diallelic" correlations, i.e., when all except the most common allele were pooled into one single composite allele. For example, even when both the multiallelic and diallelic correlations resulted in Nb estimates that were congruent with the number of parent fish that had contrib-

**Table 2.** Summary of the observed number of alleles per locus, the outcome of tests for deviations from expected Hardy–Weinberg proportions (H.–W. test), expected heterozygosity ( $H_e$ ), observed heterozygosity ( $H_o$ ), and sample size ( $n$ ) for the populations studied.

Locus <sup>a</sup>	TJA	KAR93	KAR96	KARSUP	SKJSUP95	SKJSUP96	ESR	ESRSUP95	ESRSUP96	LYS	DRO
<b>Str 15 (7)</b>											
No. of alleles	4	5	5	6	5	4	6	4	4	3	4
H.–W. test	ns	ns	ns	***	ns	*	ns	ns	ns	ns	*
$H_e$	0.720	0.709	0.737	0.645	0.713	0.665	0.568	0.563	0.538	0.621	0.355
$H_o$	0.696	0.700	0.681	0.473	0.627	0.517	0.593	0.475	0.632	0.550	0.340
$n$	46	50	72	93	59	60	54	59	76	100	100
<b>Str 60 (5)</b>											
No. of alleles	4	3	3	3	2	2	4	2	3	3	3
H.–W. test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$H_e$	0.470	0.538	0.508	0.464	0.269	0.111	0.484	0.434	0.481	0.379	0.539
$H_o$	0.417	0.500	0.542	0.398	0.317	0.083	0.426	0.559	0.500	0.460	0.450
$n$	48	50	72	93	60	60	54	59	79	100	100
<b>Str 73 (5)</b>											
No. of alleles	4	3	4	3	3	3	4	3	5	2	4
H.–W. test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	***
$H_e$	0.576	0.510	0.567	0.552	0.417	0.649	0.607	0.401	0.577	0.496	0.684
$H_o$	0.521	0.520	0.556	0.570	0.433	0.712	0.556	0.475	0.552	0.602	0.820
$n$	48	50	72	93	60	59	54	59	77	98	100
<b>SsoSL 417 (15)</b>											
No. of alleles	10	7	7	9	9	10	11	8	6	4	7
H.–W. test	ns	ns	ns	***	ns	***	ns	ns	ns	ns	***
$H_e$	0.849	0.751	0.789	0.813	0.823	0.814	0.821	0.873	0.656	0.512	0.809
$H_o$	0.896	0.740	0.833	0.548	0.797	0.661	0.611	0.966	0.662	0.590	0.840
$n$	48	50	72	93	59	59	54	58	74	100	100
<b>SsoSL 438 (8)</b>											
No. of alleles	5	5	4	6	6	4	6	4	5	3	3
H.–W. test	ns	***	ns	***	ns	ns	ns	ns	***	ns	ns
$H_e$	0.576	0.588	0.593	0.520	0.718	0.710	0.741	0.677	0.658	0.506	0.546
$H_o$	0.522	0.720	0.597	0.430	0.683	0.800	0.630	0.661	0.545	0.620	0.460
$n$	46	50	72	93	60	60	54	59	77	100	100
<b>Ssa 85 (8)</b>											
No. of alleles	6	6	6	6	8	6	6	4	5	3	4
H.–W. test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$H_e$	0.744	0.752	0.708	0.730	0.720	0.762	0.706	0.684	0.608	0.286	0.410
$H_o$	0.792	0.780	0.611	0.656	0.817	0.700	0.704	0.695	0.513	0.290	0.410
$n$	48	50	72	93	60	60	54	59	78	100	100
<b>Ssa 197 (11)</b>											
No. of alleles	7	10	9	9	7	7	8	6	6	6	6
H.–W. test	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**
$H_e$	0.836	0.755	0.786	0.789	0.775	0.708	0.758	0.778	0.703	0.400	0.674
$H_o$	0.875	0.780	0.861	0.804	0.833	0.750	0.741	0.763	0.671	0.410	0.760
$n$	48	50	72	92	60	60	54	59	76	100	100
<b>SsHaeIII14.20 (15)</b>											
No. of alleles	12	8	8	10	6	8	12	7	10	3	6
H.–W. test	ns	ns	*	***	ns	ns	ns	ns	ns	*	***
$H_e$	0.821	0.831	0.812	0.765	0.735	0.746	0.824	0.791	0.768	0.359	0.720
$H_o$	0.813	0.860	0.671	0.333	0.678	0.638	0.796	0.814	0.680	0.378	0.700
$n$	48	50	70	93	59	58	54	59	75	98	100

**Note:** See Table 1 for sample abbreviations. Table-wide significance levels were applied, using the sequential Bonferroni technique (Rice 1989) (initial  $k = 88$ ): \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

<sup>a</sup>Values in bold in parentheses are total number of alleles found per locus.

uted to the DRO and LYS samples (10 and 16 parent fish, respectively), the multi-allelic estimates of  $N_b$  tended to be high. Therefore, and because the diallelic 95% CIs were smaller, we chose to emphasise the diallelic  $N_b$  estimates.

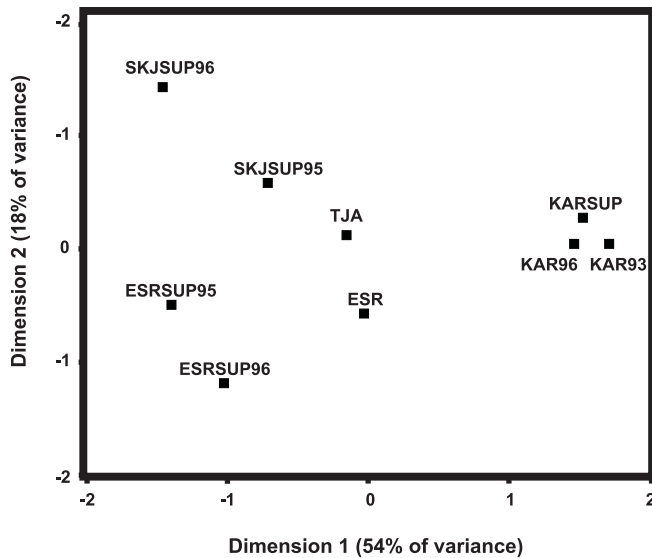
$N_b$  appeared to be high for the KARSUP and ESRSUP96 samples, whereas for the ESRSUP95 sample, it appeared to be critically low (13, with a 95% CI ranging from 6 to 29).  $N_b$  for the SKJSUP96 sample was also critically low (16,

**Table 3.**  $F_{ST}$  values between pairs of samples, based on microsatellite loci.

	TJA	KAR93	KAR96	KARSUP	SKJSUP95	SKJSUP96	ESR	ESRSUP95	ESRSUP96
KAR93	0.038***								
KAR96	0.025***	0.003							
KARSUP	0.039***	0.023***	0.019***						
SKJSUP95	0.026***	0.054***	0.042***	0.057***					
SKJSUP96	0.043***	0.105***	0.082***	0.096***	0.052***				
ESR	0.025***	0.024***	0.024***	0.038***	0.034***	0.064***			
ESRSUP95	0.030***	0.047***	0.041***	0.060***	0.026***	0.075***	0.025***		
ESRSUP96	0.036***	0.070***	0.065***	0.066***	0.043***	0.080***	0.033***	0.047***	

**Note:** See Table 1 for sample abbreviations. The significance of the values was tested by permuting individuals between samples. Table-wide significance levels were applied, using the sequential Bonferroni technique (Rice 1989) (initial  $k = 36$ ): \*\*\*,  $p < 0.001$ .

**Fig. 2.** Multidimensional scaling analysis of the matrix of pairwise Cavalli-Sforza and Edwards' (1967) distances between populations. Dimension 1 explains 54% of the variance and dimension 2 explains 18% of the variance.



with a 95% CI ranging from 6 to 48), whereas the  $N_b$  estimate for the SKJSUP95 sample was high (98) but with a very wide 95% CI (17–∞).

The tests for population bottlenecks were performed on all samples, including DRO and LYS (Table 6). The population of the Tjærøbæk River must be considered to be reasonably unaffected by most human activities and we assumed that no recent population bottlenecks had occurred. Therefore, we used the TJA sample for “calibrating” the tests for bottlenecks. When a IAM model was assumed, a significant outcome of the bottleneck tests was observed for all samples, including TJA. Conversely, when a SMM model was assumed, a significant outcome was observed in only one case, viz., the ESRSUP95 sample. This sample also exhibited the lowest  $N_b$  estimates (Table 4). When a TPM model with either 95 or 90% of all mutations being single-step mutations was assumed, significant outcomes were again observed for the ESRSUP95 sample, but there were also indications of a bottleneck in the KAR96 sample. When SMM and TPM models were assumed, there were no indications of bottlenecks in the DRO and LYS samples.

## Discussion

### Comparison of statistics for detecting low effective population sizes and loss of variability

Several statistical procedures have recently been suggested for monitoring effective population sizes and detecting population bottlenecks and loss of variability (e.g., Jorde and Ryman 1995; Cornuet and Luikart 1996; Pudovkin et al. 1996). Even though the three different approaches used all pointed to problems with a low  $N_b$  and reduced variability in the case of the ESRSUP95 sample, in other cases, the results of the three approaches did not agree. For instance, in the case of the test samples DRO and LYS, estimates of  $N_b$  from gametic disequilibria were in accordance with the number of parent fish that were actually used. At the same time, however, the bottleneck tests did not indicate a reduced effective population size in these samples. Therefore, it appears that the three methods have different strengths and limitations for genetic monitoring of supportive breeding.

Even though the randomisation test for differences in allelic diversity between samples appears to be useful, it is important to note that the samples (in this case representing propagated trout) were drawn at random from a distribution of allele frequencies that was itself determined by a sample (in this case of wild spawners). Consequently, the sample of wild spawners must be large and representative of the population as a whole. The Karup River sample from supportive breeding (KARSUP) appeared to possess more alleles at some loci than the two samples of wild spawners (KAR93 and KAR96 pooled). This could be due to the fact that the KAR93 and KAR96 samples were collected over relatively short stretches of the main river (ca. 5–10 km each), whereas the KARSUP sample consisted of offspring of trout collected over more than 20 km of both the main river and some tributaries. Therefore, the allelic composition of the KARSUP sample may in fact be more representative of Karup River trout than the KAR93 and KAR96 samples, although it should also be noted that, for several loci, significant heterozygote deficiencies were observed in the KARSUP sample, possibly owing to a Wahlund effect. This Wahlund effect could occur if fertilization occurred in batches and involved different sets of parents from different localities; this is likely the case, as the different stretches of the river were electrofished for parent fish on different days.

Estimates of  $N_b$  from gametic-phase disequilibria provide information on the crucial parameter determining loss of

**Table 4.** Tests for a reduction in the number of alleles in hatchery-reared offspring relative to wild spawners from the same population (KARSUP relative to KAR93 and KAR96 pooled; ERSUP95 and ERSUP96 relative to ESR), using a randomisation test described in Nielsen et al. (1999).

Microsatellite locus	KARSUP	ERSUP95	ERSUP96
Str 15			
<i>p</i>	1.000	0.082	0.036
No. alleles in wild source population	5	6	6
No. alleles in hatchery-reared fish	6	4	4
Str 60			
<i>p</i>	1.000	0.107	0.546
No. alleles in wild source population	3	4	4
No. alleles in hatchery-reared fish	3	2	3
Str 73			
<i>p</i>	0.241	0.319	1.000
No. alleles in wild source population	4	4	4
No. alleles in hatchery-reared fish	3	3	5
SsoSL 417			
<i>p</i>	1.000	0.207	0.0001
No. alleles in wild source population	7	11	11
No. alleles in hatchery-reared fish	9	8	6
SsoSL 438			
<i>p</i>	1.000	0.032	0.512
No. alleles in wild source population	5	6	6
No. alleles in hatchery-reared fish	6	4	5
Ssa 85			
<i>p</i>	1.000	0.108	0.542
No. alleles in wild source population	6	6	6
No. alleles in hatchery-reared fish	6	4	5
Ssa 197			
<i>p</i>	0.195	0.045	0.014
No. alleles in wild source population	10	8	8
No. alleles in hatchery-reared fish	9	6	6
SsHaeIII.14.20			
<i>p</i>	1.000	0.041	0.679
No. alleles in wild source population	9	12	12
No. alleles in hatchery-reared fish	10	7	10
<i>p</i> total	0.987	>0.001	>0.001

**Note:** See Table 1 for sample abbreviations; “*p* total” denotes the total probability obtained by combining *p* values over loci, using Fisher’s method.

**Table 5.** Effective numbers of breeders in brown trout reared for supportive breeding estimated from gametic-phase disequilibria (Waples 1991b; Bartley et al. 1992).

	KARSUP	SKJSUP95	SKJSUP96	ERSUP95	ERSUP96	DRO	LYS
<i>S</i>	92.7	59.2	59.0	58.7	75.0	100.0	99.9
Multiallelic							
$R^2$	0.009	0.013	0.018	0.031	0.015	0.039	0.018
Nb	∞	∞	292	25	197	11	44
95% CI	67–∞	55–∞	21–∞	9–149	24–∞	5–223	15–312
Diallelic							
$R^2$	0.012	0.020	0.038	0.039	0.013	0.079	0.021
Nb	463	98	16	13	∞	5	30
95% CI	33–∞	17–∞	6–48	6–29	46–∞	3–8	12–99

**Note:** *S* denotes the harmonic mean of sample sizes;  $R^2$  denotes the arithmetic mean of squared correlations between alleles at all pairs of loci; Nb denotes the effective number of breeders in one spawning season. “Multiallelic” refers to Nb estimates involving all observed alleles, whereas “diallelic” refers to Nb estimates in which the most common allele at a locus was kept while all other alleles were pooled into one composite allele.

**Table 6.** Results of tests for recent population bottlenecks, using the approach and the computer program (Bottleneck 1.2) developed by Cornuet and Luikart (1996).

Model	TJA	KAR93	KAR96	KARSUP	SKJSUP95	SKJSUP96	ESR	ESRSUP95	ESRSUP96	DRO	LYS
IAM											
No. of loci with heterozygosity excess	8	8	8	7	8	7	7	8	8	7	6
<i>p</i>	0.002	0.002	0.002	0.004	0.002	0.004	0.004	0.002	0.002	0.006	0.027
SMM											
No. of loci with heterozygosity excess	4	5	5	4	5	4	2	6	1	5	3
<i>p</i>	0.527	0.422	0.191	0.844	0.422	0.427	0.994	0.010	0.994	0.473	0.680
TPM (95%)											
No. of loci with heterozygosity excess	5	5	7	4	6	4	2	7	3	6	3
<i>p</i>	0.371	0.320	0.010	0.680	0.125	0.156	0.973	0.006	0.844	0.231	0.578
TPM (90%)											
No. of loci with heterozygosity excess	5	6	7	5	6	4	3	7	4	5	5
<i>p</i>	0.231	0.156	0.004	0.422	0.098	0.156	0.809	0.004	0.473	0.320	0.578

**Note:** IAM denotes tests assuming an infinite allele mutation model; SMM denotes tests assuming a stepwise mutation model; and TPM denotes tests assuming a two phase mutation model, with either 90 or 95% of all mutations following the SMM model. “No. of loci with heterozygosity excess” denotes the number of loci out of eight microsatellite loci studied that have higher heterozygosity values than would be expected at mutation-drift equilibrium.

variation and inbreeding, viz., effective population size itself. The statistical advantages of using multiallelic loci such as microsatellites rather than allozymes are limited, as alleles apparently need to be pooled into a diallelic state to provide reliable estimates of *N<sub>b</sub>* (see also Waples 1991*b*). This consideration must be balanced by other advantages that microsatellites possess compared with allozymes, such as the possibility for nondestructive sampling. However, a further problem has been pointed out by Waples (1991*b*) and empirically demonstrated by Bartley et al. (1992), which is that sample sizes need to be large (*n* = 100 or more). Despite the large sample sizes used here compared with most population studies based on microsatellite analysis, in some cases the upper limits of the 95% CIs could not be defined. If the intention is to identify critically small effective numbers of breeders (say, *N<sub>b</sub>* < 20), this in itself is not a problem, providing relatively narrow CIs are obtained (as in the case of the ERSUP95 and SKJSUP96 samples). However, a 95% CI ranging from a critically small value to infinity, as in the SKJSUP95 sample (95% CI of 17–∞), is not very informative, and a larger sample size would be required to distinguish signal from sampling error.

The requirements for the bottleneck test are, in principle, modest: only one sample of 20–30 individuals is required and, to achieve high power, at least five polymorphic loci, preferably more, should be studied (Cornuet and Luikart 1996; Luikart and Cornuet 1998). The actual number of loci required is a complex issue that depends on the severity of the bottleneck (i.e., the effective population size before and during the bottleneck), the time elapsed since the bottleneck, and the model of mutation assumed. As an example, Luikart and Cornuet (1998) mention that a sample size of at least 30 and analysis of at least 10 loci are required to achieve a statistical power >0.80 for detecting a 100-fold decrease in effective population size. It should be noted, however, that this example is based on a sign test, not on the more powerful Wilcoxon’s signed-ranks test applied in our study. The bottleneck test is sensitive to assumptions concerning models of mutation and this may be a problem when using microsatellites, given that this issue is still a matter of some controversy for this type of marker (Estoup and Cornuet 1999). The assumption of IAM will cause an underestimation of *H<sub>eq</sub>* and, hence, will tend to result in a significant outcome if, in fact, mutation takes place according to SMM or TPM (Cornuet and Luikart 1996). Indeed, we did observe a significant outcome in all tests that assumed IAM, even in the case of the unaffected “control” sample, TJA. The tests that assumed an SMM and, particularly, the more realistic TPM (with 5–10% of mutations involving more than one repeat unit), pointed to a bottleneck in one sample, viz., ERSUP95. This result was in accordance with the randomisation tests for loss of variation and the estimates of *N<sub>b</sub>*. The presumably low *N<sub>b</sub>* in the SKJSUP96 sample was not reflected in the bottleneck tests. However, the 95% CIs of this *N<sub>b</sub>* estimate ranged from 6 to 48, and values in the upper end of this range would not be expected to result in a significant outcome of the bottleneck tests. Perhaps more surprisingly, the bottleneck tests did not yield a significant outcome in the case of the test samples DRO and LYS, which consisted of offspring of 10 and 16 parent fish, respectively. In a survey of microsatellite variation in Danish

brown trout hatchery strains, the two strains from which the DRO and LYS samples were derived were clearly less variable than other hatchery strains and wild populations (Hansen et al. 2001). Therefore, the effective population sizes in these strains may have been low for several generations, and the use of 10 or 16 parent fish would not have created a detectable shift from mutation-drift equilibrium. This points to a general problem with applying bottleneck tests for monitoring supportive breeding; if the effective size of a population has been low for several generations before supportive breeding is initiated, a further reduction in effective population size due to bad rearing and management practices may be difficult to detect, a problem that has also been noted by Cornuet and Luikart (1996).

In conclusion, we found that none of the three statistical procedures used were ideal for monitoring loss of variation and reduced effective population sizes, and that combining information from different procedures, as in this study, would seem to be more informative. Combining information from different procedures could also solve some of the problems associated with the individual tests. For instance, strong gene flow and (or) population admixture (e.g., owing to stocking of exogenous hatchery fish) would tend to mask bottlenecks or even suggest a population expansion using the bottleneck test (Cornuet and Luikart 1996) but, at the same time, this would increase gametic-phase disequilibrium (Campton and Utter 1985) and thereby result in a low  $N_b$  estimate. Incongruent results from the two different procedures could therefore reveal if population processes other than bottlenecks had occurred.

The bottleneck test appeared to be the single most useful procedure for large-scale routine screening of supportive breeding, as only one sample is required and sample sizes and the number of loci analyzed need not be large. If the number of loci is increased, bottleneck tests could be combined with another promising development, viz., the estimation of effective population size from heterozygote excess in offspring, which is caused by random differences in allele frequencies between the sexes of the parents (Pudovkin et al. 1996; Luikart and Cornuet 1999). This procedure requires sample sizes of ca. 60 and analysis of ca. 20 loci (Luikart and Cornuet 1999). With the increasing use of automated systems for microsatellite analyses, the processing of such numbers of loci and individuals may soon be a realistic enterprise. Compared with estimating  $N_b$  from gametic-phase disequilibrium, this method requires more polymorphic loci (five alleles or more) and, thus, appears to be better suited for microsatellite loci.

### Implications for management and conservation

Supportive breeding of trout in Denmark is almost exclusively conducted on a voluntary basis by anglers' clubs, but follows guidelines based on basic conservation genetic principles (e.g., Frankel and Soulé 1981). Nevertheless, only the Karup River population appeared not to have major problems with small effective population sizes. The two samples of wild trout (KAR93 and KAR96) and the sample of reared trout (KARSUP) exhibited close genetic relationships in the MDSA of pairwise genetic distances, suggesting that little genetic drift had occurred, and the  $N_b$  estimate from the KARSUP sample was not critically low. Even though there

was statistically significant genetic differentiation between the KARSUP sample and the two samples of wild trout (KAR93 and KAR96), we believe that this reflects the circumstances of sampling, as explained previously. It is surprising, though, that the bottleneck tests on the KAR96 sample that assumed TPM suggested that a bottleneck had occurred. This outcome could reflect a recent bottleneck in the Karup River population, although a significant test would then also be expected from the KAR93 sample. Another explanation could be that the KAR96 sample contained many individuals derived from stocked trout that were from a cohort based on a small number of parent fish.

For trout from both the Skjern and Esrum rivers, the MDSA showed that different samples from the same population were genetically divergent. In the Skjern River population,  $N_b$  was too low in at least one of the samples. In the Esrum River population, supportive breeding had clearly caused a bottleneck and loss of variability. In this river, it must be considered a particularly serious problem, as in some years toxic algae are likely to eradicate large proportions of naturally reproduced trout. Consequently, stocked trout are bound to make up a large proportion of the total trout population. As a result of the effect described by Ryman and Laikre (1991), the total  $N_b$  may not be much larger than that of the propagated trout, which in 1995 may have been as small as between 6 and 29.

The factors that result in small effective numbers of breeders are not precisely known. According to the anglers' clubs responsible for rearing of trout, a relatively large number of parent fish (25–100 males and 25–100 females) are used. In principle, this should result in effective numbers of breeders of at least 50. However, the effective population size is almost always lower than the census population size, owing to variance in family size and reproductive success among individuals (Frankel and Soulé 1981). Even though several factors, such as differences in susceptibility to diseases and differences in numbers of eggs per female, could be important, we suspect that fertilisation procedures in the hatcheries may be the source of the relatively low effective population sizes. The anglers' club rearing Esrum River trout informed us that it was common procedure to add sperm from several males simultaneously to the same batch of eggs, and Withler and Beacham (1994) have demonstrated that this may lead to just one or a few males fertilising all eggs, owing to sperm competition.

The present study focused on just three trout populations subject to supportive breeding. Nevertheless, in two of the three cases, there were strong indications of reduced effective numbers of breeders. This obviously raises some concerns, and stresses the need for conducting routine genetic monitoring of supportive breeding activities and for identifying the precise factors leading to reduced effective population sizes in reared trout.

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