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Tracing the genetic origin of brown trout (*Salmo trutta*) re-colonizing the Ecker reservoir in the Harz National Park, Germany

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ABSTRACT

The Ecker reservoir and its main tributary had been free of brown trout (*Salmo trutta*) for several decades due to cumulative effects of natural and anthropogenic acidification. However, after the decline of emissions in the 1990s and the resulting rise of water pH to suitable for brown trout values, the species began to re-colonize its original habitats. In the main tributary first brown trout individuals were caught in 2008 and in the reservoir in later years as well. Stocking could be excluded in both areas. Therefore, the present study was aimed to trace the genetic origin of these brown trout by genotyping eight microsatellite loci in samples collected in the reservoir, its main tributary, potential refugia and – for comparison – from two areas downstream of the dam being physically isolated for about 70 years. Genetic variability within populations (average number of alleles per locus), genetic differentiation between populations (F_{ST} values and genetic distances), occurrence of certain alleles and results of assignment tests indicated that the Ecker reservoir was re-colonized from two sources: the Große Peseke, a small direct inflow into the reservoir, and the Fuhler Lohnsbach, a parallel flowing brook connected to the reservoir by a pipe. Genetic data also supported re-colonization of the main tributary from the reservoir but not in the opposite direction. Moreover, bottleneck effects were evident in brown trout populations upstream of the dam compared to the two populations downstream of the dam.

INTRODUCTION

The Ecker reservoir with a capacity of 13.3million·m^3 was constructed at the upper Ecker valley located in the present-day Harz National Park from 1939 to 1942. Its main tributary, the Ecker, has its spring at the western slope of the mount Brocken at an altitude of 893m. Additional minor inflows into the reservoir come from several small brooks at its eastern side and from a parallel flowing brook (Fuhler Lohnsbach) at its western side connected to the reservoir by a pipe.

Due to geological conditions and the presence of large fens near its spring the water of the Ecker and that of the reservoir has naturally low pH-values. Additional acidification caused by anthropogenic air pollution during the second half of the last century resulted in a further decline of pH-values and finally a disappearance of brown trout (*Salmo trutta*) from many brooks of the higher Harz mountains (Wüstemann 2009). A fish species survey in 1985 revealed that, with few exceptions, intact brown trout populations could only be found in the middle and lower areas of brooks originating at the Brocken massif (Wüstemann 1989; Wüstemann and Kammerad 1991). However, along with the reduction of emissions after the 1990s brown trout began to re-colonize their original habitats.

Since the Ecker reservoir and its main tributary are located within the former restricted area of the border between the two German states, fish surveys in these waters could only be conducted after the re-unification of Germany. A first survey in 1990 and sporadic surveys in 1991, 2003 and 2005 did not detect any brown trout there. In 2008, however, a few adult brown trout were caught in the main tributary to the reservoir, and

juvenile and adult individuals were detected in the Große Peseke, one of the smaller eastern inflows into the reservoir. Subsequent fishing campaigns in the main tributary in 2010 and 2011 found – in addition to a larger number of adult individuals – juvenile brown trout for the first time. Increasing numbers of brown trout were also recorded in the Ecker reservoir. In 2011, a large brown trout population was recorded in the Fuhler Lohnsbach who also was characterized by a low fish community during the times of acidification.

Stocking of brown trout into the Ecker reservoir or its main tributary can be excluded since they are located in formerly restricted areas that became part of the Harz National Park later on. Thus, natural re-colonization could be assumed and the present study was aimed at tracing the genetic origin of these brown trout by microsatellite analyses of samples collected in the reservoir, its main tributary, potential refugia (Große Peseke and Fuhler Lohnsbach) where brown trout could have survived acidification and – for comparison – from two areas downstream of the dam being physically isolated for about 70 years since the dam construction.

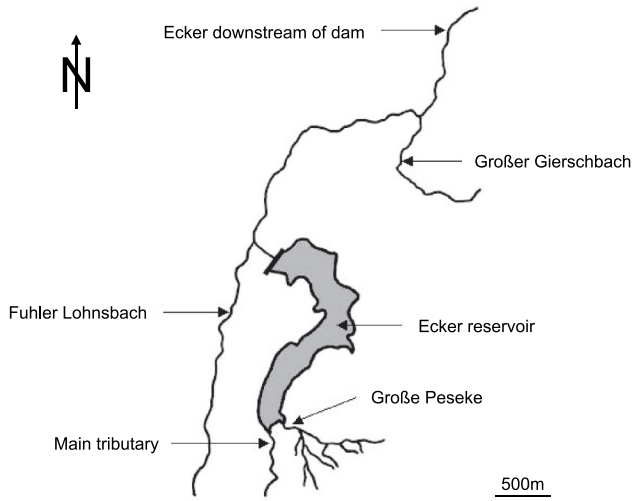

Figure 1. Map of brown trout sampling locations.

MATERIAL AND METHODS

Fish sampling

Brown trout were caught by gill nets in the Ecker reservoir and by electro fishing at other five locations in 2010 and 2011 (Figure 1): Ecker reservoir (n=25) and its main tributary (n=20), Fuhler Lohnsbach (n=35), Große Peseke (n=19), Ecker downstream of dam (n=31), and Großer Giersbach (n=33). Fin clips were taken and stored in pure ethanol.

Microsatellite genotyping

Genomic DNA was isolated from the fin clips using the peqGOLD Tissue DNA Mini Kit (Peqlab Biotechnologie). The reaction mixtures for PCR amplification of eight microsatellite loci (Table 1) on a Mastercycler Gradient apparatus (Eppendorf) consisted of 1.5μl of 10·PCR buffer with $(NH_4)_2SO_4$ (MBI-Fermentas), 1.2μl of 25mM

Table 1. Primer sequences (F=forward; R=reverse), PCR programs and references for the eight microsatellite loci genotyped in brown trout.

Microsatellite	Primer sequences	PCR program	Reference
MST-60	F: gcggtgtgcttgtcaggtttc R: gtcaagtcagcaagcctcac	A	Presa and Guyomard (1996)
OMM1310	F: cgcgtgacagtgaaaagtaatagc R: ttatcattccctaccaatcgatcc	B	Palti et al. (2002)
Ssa85	F: gaggtgggtcctccaagctac R: gacccgctcctcacttaatc	C	O'Reilly et al. (1996)
Sco204	F: gctaaggatggtcactcat R: gcaacacagaaatgtaactct	A	DeHaan and Ardren (2005)
MST-15	F: gtgcaggcagacggatcaggc R: gaatcctctactgaagggatttgc	C	Presa and Guyomard (1996)
SsaA86	F: tctcccagtggttctagatgag R: ggagctaaacttcaaagcacag	C	King et al. (2005)
SSOSL85	F: gtgtggatttttgtattatgtta R: gatacatttcctcctcattcagt	B	Slettan et al. (1995)
Ssa410UOS	F: ggaaaataatcaatgctgctggtt R: gctacaatctggactatcttcttca	D	Cairney et al. (2000)

Table 2. Details of the four PCR programs used to amplify the eight microsatellite loci of brown trout.

A	B	C	D
95°C; 5min	95°C; 5min	95°C; 5min	95°C; 5min
5 cycles:	5 cycles:	5 cycles:	5 cycles:
95°C; 1min	95°C; 40sec	95°C; 40sec	95°C; 1min
54°C; 1min	50°C; 40sec	53°C; 40sec	58°C; 1min
72°C; 1min	72°C; 40sec	72°C; 40sec	72°C; 1min
35 cycles:	30 cycles:	30 cycles:	35 cycles:
90°C; 1min	90°C; 40sec	90°C; 40sec	90°C; 1min
54°C; 1min	50°C; 40sec	53°C; 40sec	58°C; 1min
72°C; 1min	72°C; 40sec	72°C; 40sec	72°C; 1min
72°C; 7min	72°C; 10min	72°C; 10min	72°C; 7min

$MgCl_2$, 1.2μl of 1.25mM dNTPs, 0.3μl of each primer (10pmol·μl^{-1}), 3.0μl genomic DNA, 0.1μl *Taq* DNA polymerase (5Uμl^{-1}; MBI-Fermentas) and sterile water up to a final volume of 15.0μl. PCR programs are described in Table 2. Forward primers of each pair were labelled with one of three WellRED dyes (Sigma-Aldrich) to enable recording of microsatellite genotypes on a capillary sequencer (CEQ 8000, Beckman Coulter) using the Fragment Analysis module of the CEQ 8000 Genetic Analysis System, version 7.0.

Data analyses

Groups of brown trout individuals from each of the six sampling locations were treated as distinct populations. Initially, all microsatellite genotypes were tested with the program MICRO-CHECKER (van Oosterhout et al. 2004) for evidence of null alleles, large allele dropout and/or scoring errors due to stuttering. The GENEPOP program package (Raymond and Rousset 1995) was then used to estimate within-population genetic variability parameters (total and average allele numbers, number of private alleles, observed and expected heterozygosities) and to test for deviations from Hardy-Weinberg equilibrium (probability test: estimation of exact *P*-values by the Markov chain method). Differences in variability parameters between populations were evaluated using the t-test. The genetic differentiation between populations was assessed by calculating pairwise F_{ST} values (Weir and Cockerham 1984) and genetic distances D_A (Nei et al. 1983) with the program MICROSATELLITE ANALYSER (Dieringer and Schlötterer 2003). Significance levels of F_{ST} values were adjusted using the sequential Bonferroni correction (Rice 1989). In order to construct a UPGMA tree illustrating the genetic relationships between populations bootstrapping with 1,000 replicates was performed on D_A distances. The resulting set of 1,000 distance matrices was then taken to run the NEIGHBOR and CONSENSE modules of the PHYLIP program package (Felsenstein 1993). The publication-ready tree was plotted using the MEGA program (Kumar et al. 2004). Finally, the interpopulational dispersal of brown trout individuals was assessed by an assignment test (GeneClass program; Cornuet et al. 1999) using the Likelihood and Bayesian method and algorithm.

RESULTS

Genetic variability within the brown trout populations

All eight microsatellite loci were found to be polymorphic in all six brown trout populations examined. MICRO-CHECKER did not indicate any evidence for large allele drop out or scoring errors. The total number of alleles over all eight loci amounted to 68, with a total number of alleles per locus ranging from two (*MST-60*) to 18 (*Ssa410UOS*). At the population level, the average number of alleles per locus ranged from 3.62 (Große Peseke) to 7.12 (Ecker downstream of dam) (Table 3). Statistically significant differences in the average number of alleles per locus were only found between the brown trout with the highest variability (Ecker downstream of dam: 7.12 alleles per locus) and the two populations with the lowest variability (Große Peseke: 3.62; main tributary: 3.88). The low value of 3.88 observed in brown trout from the main tributary was probably caused by the presence of null alleles at the two loci *OMM1310* and *Ssa85* as indicated by MICRO-CHECKER test results. In contrast, MICRO-CHECKER did not provide any indication for null alleles in brown trout from the Große Peseke what could have explained their low variability of 3.62 alleles per locus.

The average values for observed heterozygosity (H_O) ranged from 0.526 (Große Peseke) to 0.698 (Ecker downstream of dam) and those for expected heterozygosity (H_E) from 0.477 to 0.663 in the same two populations (Table 3). However, all differences between populations in both parameters were statistically non-significant.

Table 3. Average number of alleles per locus (n), observed (H_O) and expected (H_E) heterozygosities at eight microsatellite loci of six brown trout populations. R=Ecker reservoir; F=Fuhler Lohnsbach; T=main tributary; P=Große Peseke; E=Ecker downstream of dam; G=Großer Giersbach.

Parameter	R	F	T	P	E	G
n	4.38	4.25	3.88	3.62	7.12	5.25
H_O	0.582	0.589	0.544	0.526	0.698	0.614
H_E	0.586	0.540	0.565	0.477	0.663	0.562

The brown trout from the Ecker reservoir, Fuhler Lohnsbach and Große Peseke were found to be in Hardy-Weinberg equilibrium. In contrast, the brown trout from the main tributary, Ecker downstream of dam and Großer Giersbach displayed highly significant deviations from equilibrium. In case of the brown trout from the main tributary this deviation could have been related to the presence of null alleles at the two loci *OMM1310* and *Ssa85*.

Genetic differentiation between the brown trout populations

Out of the 68 microsatellite alleles, 20 were shared by all six brown trout populations and 21 were private alleles occurring in one population only. Most of the private alleles (15) were detected in brown trout from the Ecker downstream of dam what could be attributed to the high genetic variability of this population. No private alleles were observed in brown trout from the Ecker reservoir and its main tributary. One private allele was found in brown trout from the Große Peseke, two in those from the Fuhler Lohnsbach, and three in those from the Großer Giersbach. The number of private alleles increased to 26 if the two populations from the Ecker downstream of dam and Großer Giersbach were pooled. This already indicates a remarkable genetic differentiation between the two brown trout populations downstream of the dam and the remaining four populations as well as a close relationship of the brown trout from the Ecker reservoir and its main tributary.

Table 4. Pairwise F_{ST} values (above diagonal; non-significant values in bold) and genetic distances (below diagonal) between six brown trout populations. Population labels see Table 3.

*	R	F	T	P	E	G
R	*	0.028	**0.011**	**0.022**	0.080	0.107
F	0.062	*	0.046	0.034	0.105	0.134
T	0.029	0.073	*	0.055	0.089	0.137
P	0.064	0.064	0.063	*	0.121	0.147
E	0.188	0.220	0.187	0.204	*	0.071
G	0.194	0.207	0.211	0.211	0.148	*

More precise information on the genetic differentiation is provided by the pairwise F_{ST} values and genetic distances (Table 4). Statistically non-significant genetic differentiations were only observed between the brown trout from the Ecker reservoir and its main tributary as well as between the brown trout from the Ecker reservoir and the Große Peseke. All remaining pairwise comparisons revealed significant or highly significant differentiations. The shortest genetic distance was found between the brown trout from the Ecker reservoir and its main tributary. The generally largest genetic distances from all other populations were measured for brown trout from the Ecker downstream of dam and from the Großer Giersbach.

The UPGMA tree (Figure 2) showed two clearly separated groups with high bootstrap support (100%) to each of them. The first group consisted of brown trout from the Ecker reservoir, its main tributary, the Große Peseke and the Fuhler Lohnsbach, including a sub-group with high bootstrap support (99.3%) composed of brown trout from the Ecker reservoir and its main tributary. The second group was formed by the two populations from downstream of the dam: Ecker and Großer Giersbach.

The results of the assignment tests (Table 5) generally corresponded with the degree of genetic differentiation between the populations. The highest proportions of correctly assigned individuals were recorded in brown trout from the Ecker downstream of dam (100.0%) and from the Großer Giersbach (93.9%) who also displayed the strongest differentiation from each other as well as from the remaining four populations (see F_{ST} values in Table 4). The lowest proportions of correctly assigned individuals were found in brown trout from the Ecker reservoir (64.0%) and its main

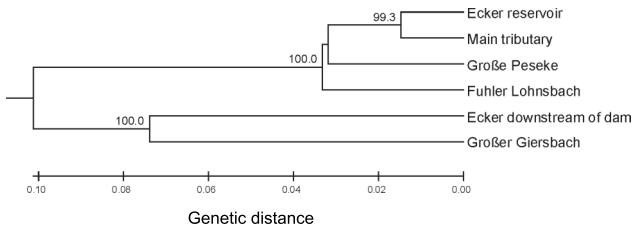

Figure 2. UPGMA tree based on D_A genetic distances between the six brown trout populations.

tributary (60.0%) what could be explained by their non-significant differentiation from each other and their close genetic relationships with brown trout from the Große Peseke and the Fuhler Lohnsbach.

DISCUSSION

When interpreting the results of the microsatellite analyses in order to trace the genetic origin of brown trout that re-colonized the Ecker reservoir and its main tributary the following assumptions have to be considered: (1) all microsatellite alleles actually occurring in the six populations have been gathered by the present sampling, (2) no new alleles were created by mutations, and (3) the present genetic structure of brown trout populations is identical or at least comparable to that at the time of re-colonization. Although these assumptions are possibly not all met in reality (sample size in some cases might be too low to detect very rare alleles; small but expanding founder populations might possess instable genetic structures) the following scenario seems to be feasible, in particular in connection with known environmental data (pH-values):

Table 5. Results of the brown trout assignment tests. Population labels see Table 3.

Population	Total number	Number of individuals assigned into:						Correctly assigned (%)
		R	F	T	P	E	G	
R	25	16	5	3	1	0	0	64.0
F	35	2	29	0	4	0	0	82.9
T	20	2	1	12	4	0	1	60.0
P	19	0	0	4	15	0	0	78.9
E	31	0	0	0	0	31	0	100.0
G	33	1	1	0	0	0	31	93.9

- The close relationship (non-significant genetic differentiation) of brown trout from the Ecker reservoir and from the Große Peseke supports the hypothesis that this brook due to more suitable pH-values might have served as a refuge during the acidification of the reservoir. The subsequent re-colonization of the Ecker reservoir, however, could not have originated from the Große Peseke alone because the microsatellite locus *OMM1310* possessed two alleles and the locus *Ssa410UOS* one allele that were found in brown trout from the reservoir but could not be detected in those from the Große Peseke. Since these alleles were present in brown trout from the Fuhler Lohnsbach at least a few individuals must have immigrated from there into the reservoir as well. Vice versa, there was one allele at the locus *Ssa410UOS* being shared among brown trout from the Ecker reservoir and the Große Peseke but missing in individuals from the Fuhler Lohnsbach. Further indication for the re-colonization of the Ecker reservoir from two different sources is provided by the fact that the average number of alleles per locus was tendentially higher in brown trout from the reservoir (4.38) than from the Große Peseke (3.62) and the Fuhler Lohnsbach (4.25).
- Then, the re-colonization of the main tributary from the Ecker reservoir (but not in the opposite direction) is supported by a tendentially higher average number of alleles per locus in brown trout from the reservoir (4.38) than from its main tributary (3.88) and their non-significant genetic differentiation. Moreover, these two brown trout populations displayed the shortest genetic distance (0.029) between the six populations examined.

Independent from this scenario it should also be considered that the re-colonization of the Ecker reservoir might not have been a single event but could have occurred several times and simultaneously or consecutively from both possible sources and over several generations. Moreover, re-emigrations of brown trout from the Ecker reservoir into the Große Peseke can also not be excluded.

Assuming that the brown trout populations downstream of the dam represent an undisturbed, natural population of this water system, it could be expected that the historical populations at the location of the present-day reservoir and upstream from it should have possessed a similarly high genetic variability. The significant lower variability (measured as the average number of alleles per locus) of the brown trout from

the main tributary and the Große Peseke should thus be interpreted as a bottleneck or founder effect caused by a combination of factors: shrinking of suitable habitat → reduction of population size → occurrence of genetic drift (loss of rare alleles by chance). A genetic monitoring at larger time intervals (after several generations) is therefore recommended in order to counteract if their genetic variability should decline further. Under such circumstances controlled stocking of brown trout from genetically similar populations might be a reasonable measure to recover genetic variability and thereby contribute to a long-term adaptability of populations to changing environments.

The two major groups into which all six brown trout populations were clustered according to their topographical position (upstream or downstream of the dam) clearly reflect the present-day reproductive isolation and disabled gene flow caused by the dam. However, the also significant differentiation between the two populations downstream of the dam suggests that at least parts of the observed genetic differences were already existing before the dam construction and that brown trout shows a small-scale spatial population structure in the water system examined.

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