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Spatiotemporal analysis shows stable genetic differentiation and barriers to dispersal in the Eurasian perch (*Perca fluviatilis* L.)

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ABSTRACT

Background: Recently, unexpected or cryptic barriers to gene flow causing genetic discontinuities have been found in a number of animal taxa, even in apparently highly connected areas such as aquatic environments.

Goal: Investigate the temporal stability of previously documented microgeographic genetic structure in a fish.

Organism: Eurasian perch (*Perca fluviatilis* L.)

Methods: We sampled four locations over a period of 2 years. We used six microsatellites to investigate population differentiation. We compared within-year to between-year differentiation.

Results: The significant genetic differentiation found between locations in 2004 was still present in 2006. The strongest barriers to gene flow in the lake were consistent over both sampling periods. Furthermore, temporal differentiation existed within each site between the years. Populations of perch appear to cluster in different patches in the lake that harbour genetically differentiated groups of fish. Hence, limited migration and barriers to dispersal can persist over time, even at a very small geographical scale and in an open aquatic environment.

Keywords: barriers to gene flow, microsatellites, *Perca fluviatilis* L., small-scale genetic divergence, spatiotemporal stability.

INTRODUCTION

The view of the aquatic environment as being open and homogenous, containing few physical barriers to gene flow, has recently been challenged. Studies have shown that despite the possibility for extensive gene flow, population differentiation can arise (Adams *et al.*, 2006; Barluenga *et al.*, 2006; Duftner *et al.*, 2006; Nicholls *et al.*, 2006; Bergek and Björklund, 2007; Thuesen *et al.*, 2008). Identifying barriers to dispersal, exploring what they are and whether they are stable over

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time is of great importance for understanding the evolution and extent of population structure. Stable structuring between adjacent populations might be expected to facilitate inter-population genetic and ecological differentiation and even speciation (Coyne and Orr, 2004). In small isolated populations in particular, drift and local adaptation will have important effects on gene frequencies and lead to increased divergence. Hence, understanding the stability of reduced gene flow and population structure is very important from both an evolutionary and conservational perspective. Several mechanisms, both related to the environment and innate behavioural ones, could give rise to such barriers. In fish, landscape features such as ocean currents and habitat discontinuities have been shown to affect the level of differentiation (Grant and Bowen, 1998; Knutsen *et al.*, 2003; Jørgensen *et al.*, 2005; Ruzzante *et al.*, 2006; Leclerc *et al.*, 2008). In addition, behavioural mechanisms such as formation of kin groups have been shown to affect the genetic population structure (Gerlach *et al.*, 2001).

When genetic variation is investigated on a temporal scale, there are both biological and non-biological factors that can affect the outcome and give opposing results. First, species with a larval phase might have very high fecundity but also high mortality in the early life stages (Hedgecock, 1994). Hence, for a given year, most of the recruited young may be from very few parents because of a 'sweepstake-chance matching of reproductive activity' (Hedgecock, 1994). This would thus result in both a high variance in the number of progeny and a very small effective population size, N_e, a phenomenon called the 'Hedgecock effect' (Waples, 1998). This could then result in significant temporal variation of allele frequencies if different cohorts have been sampled. Hence investigating genetic differentiation over time is very important to avoid biased estimates. Second, non-biological events, such as environmental variability, might lead to unpredictable patterns of genetic differentiation (Pertoldi *et al.*, 2007; Ranta *et al.*, 2008), thereby influencing temporal stability. Thus, to get an unbiased estimate of spatial population differentiation, it is crucial to sample and analyse population structure over time (Ryman, 1997; Waples, 1998).

Relatively few studies have addressed the temporal stability of allele frequencies, and they have yielded contrasting results. In some studies, the genetic differentiation was stable over time (Nielsen *et al.*, 1997, 1999; Tessier and Bernatchez, 1999; Hansen, 2002), whereas in others a genetic admixture between years was observed (Hoarau *et al.*, 2002; Barcia *et al.*, 2005; Dannewitz *et al.*, 2005). Consequently, given the importance for an evolutionary as well as conservational perspective, this is an area of research that deserves more attention.

We have investigated temporal stability of genetic differentiation at small scales by studying the perch (*Perca fluviatilis* L.). Perch spawn in late spring with the female shedding her eggs in shallow water on vegetation or substrates, followed by fertilization by nearby males (Thorpe, 1977). Fecundity is high; 300,000 eggs per female have the potential to be fertilized by several males (Thorpe, 1977). This high fecundity and the probable high mortality could lead to a high variance in reproductive success and low N_e, in accordance with the 'Hedgecock effect' mentioned above. The duration of the pelagic larval phase is approximately 3–4 weeks, after which the juvenile perch swim back into the littoral zone. The species is considered sedentary (Kipling and Le Cren, 1984), returning to their natal site to spawn. Behrmann-Godel and Gerlach (2008) have shown intrinsic genetic incompatibilities between two genetically differentiated populations within the same lake. This suggests a potential for post-zygotic isolation in this species, and further implies that temporally stable differentiation should be present. A recent study demonstrated that populations of perch were genetically differentiated within a single, small lake (Bergek and Björklund, 2007), with barriers to dispersal present at unexpectedly short distances. The purpose of the present

study was to assess the stability of the genetic differentiation among contemporary replicates previously sampled 2 years earlier (2004) in the study by Bergek and Björklund (2007). In addition, we wished to determine whether the cryptic barriers to dispersal found within the lake in 2004 remained, in the same places, 2 years later. We expected the spatial genetic difference to be stable over time unless the system is heavily affected by stochastic events such as temporal variation in gene frequencies between cohorts, a high degree of dispersal or environmental variability.

MATERIALS AND METHODS

Sampling

We caught 177 fish at four locations (Location 1, $n = 45$; Location 3, $n = 44$; Location 5, $n = 45$; Location 9, $n = 43$) in Lake Erken during August 2006 (Fig. 1B). These locations had been sampled previously during August 2004 (Bergek and Björklund, 2007). Total length (mm) was recorded to approximate age classes. Differences in length within both spatial and temporal samples were investigated by using a *t*-test. Fin-clips were taken for DNA analysis and were stored in 95% ethanol for further analysis. The total genetic data are based on the 177 fish caught in 2006 and 119 fish (same four locations) caught in 2004 (see Appendix).

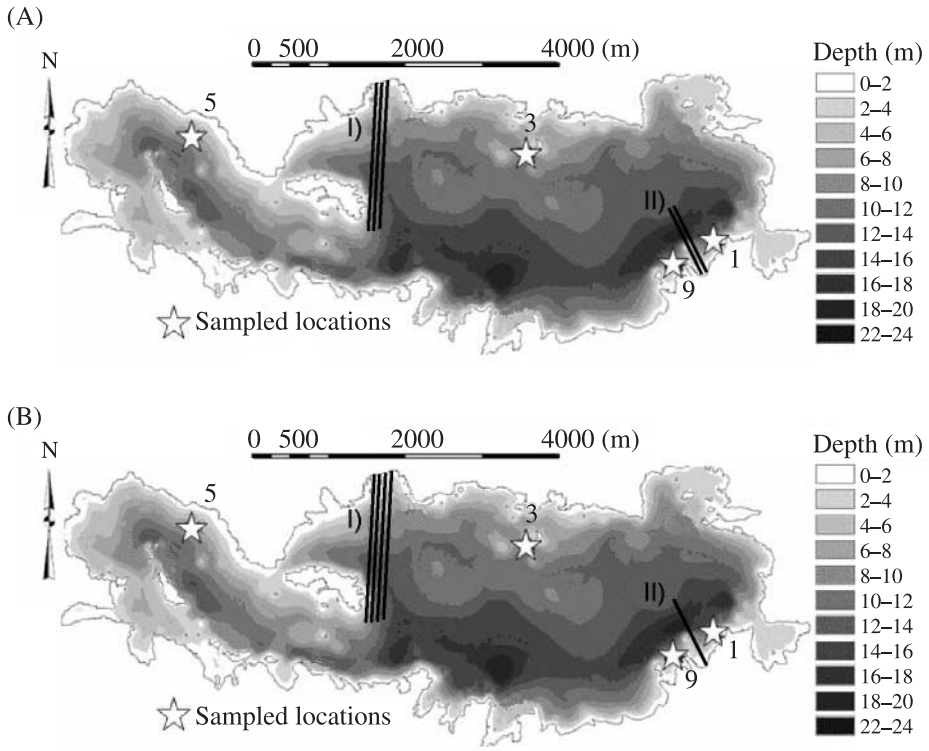

Fig. 1. Sampled locations (Locations 1, 3, 5, and 9) in Lake Erken from (A) 2004 and (B) 2006. Lines represent the position and number of detected barriers to gene flow identified by the software BARRIER (Manni *et al.*, 2004).

DNA extractions

We extracted DNA with a Chelex proteinase K kit according to Walsh *et al.* (1991), following the manufacturer's protocol. Each fish was genotyped using six dinucleotide microsatellite loci: Svi4, Svi7, and Svi10 developed for walleye (Wirth *et al.*, 1999), and Pfla1, Pfla6, and Pfla9 developed for yellow perch (Leclerc *et al.*, 2000). The PCR conditions used are described by Wirth *et al.* (1999) and Leclerc *et al.* (2000). One microlitre of PCR product was mixed with 20 µl TAMRA (Applied Biosystems) and 90 µl Formamide (Amersham Biosciences). Fragments were separated on 1% polyacrylamide sequencing gels under denaturing conditions by using an ABI 377 Prism automated sequencer (Applied Biosystems). The sizes of the fragments were inferred by using TAMRA as standard and genotype data were generated by using the GeneScan Analyses 3.1.2 software program (Applied Biosystems).

Hardy-Weinberg equilibrium (HWE)

Potential genotyping errors, such as allelic dropouts, stuttering or null alleles, were analysed using the program MICRO-CHECKER version 2.2.1 (Van Oosterhout *et al.*, 2004). Linkage disequilibrium was tested with a likelihood ratio test as implemented in GENEPOP version 1.2 (Raymond and Rousset, 1995). The same software was used to calculate observed (H_O) and expected (H_E) heterozygosities. Deviation from Hardy-Weinberg equilibrium was calculated using χ^2-tests of observed and expected heterozygosity for each locus and location. In addition, estimation of F_{IS}-values and testing for heterozygote deficiencies and departure from HWE was performed using FSTAT version 1.2 (Goudet, 1995). In total, 960 randomization tests were performed to determine the proportion of randomizations that gave a larger F_{IS}-value than the observed. FSTAT version 1.2 (Goudet, 1995) was also used to obtain gene diversity. Differences among temporal replicates in gene diversities were tested with a Mann-Whitney *U*-test.

Genetic differentiation

Genetic differentiation among all populations and between population pairs at both spatial and temporal scales was estimated by F_{ST}-values, obtained by GENEPOP version 1.2 (Raymond and Rousset, 1995) after the method of Weir and Cockerham (1984). A hierarchical analysis of variance (ANOVA) was done in ARLEQUIN 2.0 (Schneider *et al.*, 2000), assessing significance from 1000 permutations, to evaluate the degree of differentiation among temporal samples. The hierarchy levels were set as follows: among populations sampled at the same year, and between temporal replicates of the same population. We also estimated the power of potential genetic differentiation by using the software POWSIM (Ryman and Palm, 2006).

Geographical barriers

To determine whether the most prominent barriers to dispersal found in the lake in 2004 were situated in the same locations 2 years later, we used the software BARRIER (Manni *et al.*, 2004). The geographical coordinates for each location were connected by Delaney triangulation. Barriers between each distance in the triangulation were identified using Monmonier's maximum distance algorithm (for further details, see Manni *et al.*, 2004). F_{ST}-values for six loci (locus Svi4 was not included because we lacked data for three of the locations

in 2004) were used separately to test for discontinuity. The strength of the barrier was determined by the number of loci that supported the discontinuity.

Migration rates

To obtain an estimate of recent migration rates among sampled locations, we used the software BAYESASS (Wilson and Rannala, 2003). This was done for the 2006 sample and compared with the estimates for 2004 presented in Bergek and Björklund (2007). We also estimated the migration rates temporally. The model was run with default parameters.

Effective population size

Temporal genetic studies can be used to estimate effective population size (Berthier *et al.*, 2002). However, since our species has overlapping generations, demographic data would be required to obtain an unbiased estimation of effective size (Jorde and Ryman, 1995). With no such information, we used the method of Xu and Fu (2004), where $\theta = 4N_e\mu$, to obtain an approximate estimate of effective population size. This analysis assumes knowledge of mutation rate (μ), which may differ between loci, and one should therefore be careful when drawing conclusions from the results. Here we used the assumed mutation rate (μ), which for microsatellites is 10^{-3} (Weber and Wong, 1993; Jarne and Lagoda, 1996). To increase the validity of the results, we also fitted a model using a higher mutation rate parameter: 5×10^{-3}.

Relatedness

Sampling of kin groups could give rise to a false signal of population structure. To exclude this possibility, we calculated pairwise relatedness (r) values, using the method of Lynch and Ritland (1999). A simulated data set consisting of four populations, seven loci, and the same level of genetic variability as in our study set was created. Next, the r-values among these non-kin individuals were calculated to get the distribution of relatedness in the simulated data set. To evaluate the presence of kin groups within each sample from our data set, the r-values were calculated and the distribution was compared to the total whole real sample using a Kolmogorov-Smirnoff test. To establish if individuals in each location are more related than a random group taken from the whole lake, the mean r-value for each location for the N individuals was calculated and compared to the mean r-value obtained by sampling N individuals randomly from the total real sample. This was repeated 5000 times for each location and the proportion of times a mean r-value as high as or higher than the observed value was recorded (P-value in Table 2). Finally, we tested if the distribution of r-values had a long right-hand tail, indicating kin groups, by comparing the proportion of r-values above 0.2 at each location to the same proportion in the random samples (for further details, see Bergek and Björklund, 2007).

RESULTS

Length

There was a temporal difference in body length at Location 5 (t-test, $t = 2.8$, $P = 0.007$), with larger fish in the sample from 2006. All other locations were temporally and spatially similar (t-test, $P > 0.05$).

HWE and gene diversity

The temporal stability of genetic differentiation was assessed using six loci. Previous estimates based on eight loci from the 2004 study were re-calculated using these six loci; no significant change was observed. The mean numbers of alleles per locus and χ^2-values are presented in the Appendix. Departure from HWE was found in 6 of 46 comparisons (13%). In those cases, there was an excess of homozygotes. Analysing the data with MICRO-CHECKER showed that the homozygote excess was most likely due to the presence of null alleles. Therefore, we adjusted for null allele homozygotes following Van Oosterhout *et al.* (2004) on a locus-by-locus basis by coding them as missing data based on the adjusted genotypes by MICRO-CHECKER version 2.2.1 (Van Oosterhout *et al.*, 2004). The adjusted genotypes were used to calculate test statistics, which were compared to the original values. However, using the adjusted data set with corrected genotypes for null alleles did not change the overall results. The excess of homozygotes was also shown as elevated F_{IS}-values ranging from 0.149 up to 0.268 (Table 1). Analyses with the adjusted data set gave lower F_{IS}-values, but the overall F_{IS}-values still remained significant (Table 1). Genetic diversity estimates showed no changes among sampled locations between temporal replicates (Mann-Whitney *U*-test, $U = 238$, $P > 0.05$).

Genetic differentiation

The genetic differentiation found in 2004 ($F_{ST} = 0.025$, $P < 0.05$) appears to be stable, since the current study also revealed a significant global F_{ST}-value ($F_{ST} = 0.025$, $P < 0.05$) and all pairwise comparisons were significant ($P < 0.05$). A power analysis indicated sufficient power to detect the level of genetic differentiation observed, since a true F_{ST}-value of 0.005 would be detected with a probability of 99% with the observed allele frequencies. The variance component among the temporal samples ($\Phi sc = 0.014$, $P < 0.001$) does not exceed the spatial variance ($\Phi sc = 0.010$, $P < 0.001$).

Geographic barriers

Barriers to gene flow were situated in the same position as in the 2004 study (Bergek and Björklund, 2007) (Fig. 1A, B), indicating temporal persistence of spatial genetic differentiation. The only

Table 1. F_{IS}-values per locus and location obtained from FSTAT version 1.2 (Goudet, 1995)

Locus	Location 1	Location 3	Location 5	Location 9
Svi4	−0.028 (−0.028)	0.077 (0.077)	0.079 (0.079)	**0.150** (0.150)
Svi7	**0.372** (0.091)	**0.303** (0.066)	**0.299** (**0.106**)	**0.301** (**0.135**)
Svi10	0.090 (0.090)	0.026 (0.026)	0.045 (0.045)	0.106 (0.106)
Pfla1	**0.233** (0.024)	0.063 (0.063)	**0.238** (**0.153**)	**0.485** (0.036)
Pfla6	**0.412** (0.010)	0.089 (0.089)	**0.285** (−0.001)	**0.285** (**0.208**)
Pfla9	**0.282** (0.108)	**0.287** (0.105)	**0.149** (**0.096**)	**0.249** (**0.153**)
All	**0.244** (**0.051**)	**0.149** (**0.073**)	**0.231** (**0.084**)	**0.268** (**0.128**)

Note: Observed F_{IS}-values obtained from the adjusted data set for null alleles according to the method of Brookfield (1996) are shown in parentheses (Van Oosterhout *et al.*, 2004). **Bold** numbers indicate statistical significance ($P < 0.05$) after 1000 randomizations.

difference in 2006 was an increase in the strength of barrier (I) with one additional supporting locus, and a decrease in the strength of barrier (II) with the loss of one supporting locus.

Migration rates

Based on estimates from BAYESASS, recent immigration rates in the current study are quite low. The only locations expressing significant migration rates are from Location 5 to 1 (mean = 0.22, S.D. = 0.10), Location 1 to 5 (mean = 0.056, S.D. = 0.094), Location 1 to 9 (mean = 0.061, S.D. = 0.087), Location 3 to 9 (mean = 0.067, S.D. = 0.037), and Location 5 to 9 (mean = 0.18, S.D. = 0.087). These rates are all within the same range of estimates as in the 2004 study (Bergek and Björklund, 2007). A high proportion of individuals were derived from their own population ($P > 0.95$), suggesting that most of the locations are isolated from each other, at least with respect to first- and second-generation immigrants (see Wilson and Rannala, 2003).

Effective population size

In the present study, θ ranged from 0.91 (S.E. = 0.29, Location 1) to 1.13 (S.E. = 0.51, Location 9). Assuming a mutation rate of 10^{-3}, we have a population size of 227 (range 120–370, Location 1) to 283 (range 103–1041, Location 9) individuals. Estimates assuming a mutation rate of 5×10^{-3} gives an effective population size of 181 (range 96–196, Location 1) to 226 (range 82–433, Location 9).

Relatedness

Relatedness values were low but significantly higher than any two random individuals in the total sample (Table 2). The proportion of positive *r*-values was significantly larger than expected from the random data set in one of the locations (Table 2), indicating a high relatedness, but not pure kin groups.

DISCUSSION

This study provides evidence for stable genetic differentiation at a very small spatial scale in the Eurasian perch. Barriers present in the lake in 2004 (Bergek and Björklund, 2007) were observed in the same locations 2 years later, indicating restricted gene flow over time. The

Table 2. Mean (*r*), standard deviation (S.D.), and the proportions of times a higher value than the observed value was found in the randomizations (*P*)

Location	Mean *r*	S.D.	*P*	*P* (Kolmogorov)	% > 0.2	*P*
1	0.016	0.15	0.0028	0.036	11.1	0.014
3	0.029	0.17	<0.001	$0*10^{-12}$	13.7	<0.001
5	0.009	0.15	0.011	0.0006	10.0	0.164
9	0.014	0.17	0.007	0.039	11.0	0.026

Note: *P* (Kolmogorov) refers to the Kolmogorov-Smirnoff test of the distribution of relatedness values compared to the total population. The observed proportion of relatedness values above 0.2 (% > 0.2) and the number of times a higher proportion was found in the randomizations (*P*).

hierarchical analysis of molecular variance revealed that the proportion of the total genetic variance explained by differences between temporal samples within locations was of a similar magnitude as the proportion owing to spatial differences. Hence, there is a stable spatial structure at a certain geographical patch size in the lake, but also a large temporal variation in allele frequencies at each location. This could be due to the presence of several small populations/schools, each belonging to a specific region (patch) of the lake, and the fact that different schools were sampled within each location at the two sampling events.

Previous studies have revealed disparate patterns when population differentiation is studied temporally. For example, in the shrimp [*Farfantepenaeus notialis* (Robainas Barcia *et al.*, 2005)], eel [*Anguilla anguilla* L. (Dannewitz *et al.*, 2005)], and plaice [*Pleuronectes platessa* L. (Hoarau *et al.*, 2002)] measures of genetic differentiation revealed genetic homogeneity when sampled over several years. In contrast, in the cod [*Gadus morhua* (Jónsdóttir *et al.*, 2001; Ruzzante *et al.*, 2001)], salmon [*Salmo salar* (Tessier and Bernatchez, 1999; Potvin and Bernatchez, 2005)], trout [*Salmo trutta* (Laikre *et al.*, 2002; Jensen *et al.*, 2005)], and sea bream [*Diplodus sargus* (Lenfant and Planes, 2002)], for example, a stable population structure was observed. The possibility of temporally stable structure might differ between organisms, depending on the geographical area, dispersal possibilities, and how much it is affected by environmental stochasticity. For example, in certain geographical areas, storms could have a huge impact, promoting dispersal/drifting of young fish/larvae, thus leading to population admixture. However, the lake studied here is located in an area not strongly affected by storms and currents, which might make it easier for stable genetic differentiation to arise.

Leclerc *et al.* (2008) showed that in the closely related yellow perch (*Perca flavenscens*), the distance along the water was not the most important factor for restricting gene flow. Instead, they found that the genetic discontinuity was associated with physical barriers and distinct physico-chemical water masses in the study area. This study was performed in a river where distance and barriers are easier to define. In our study, however, no environmental data have been collected and thus we cannot speculate as to whether the environment has any impact on genetic structuring in the lake. In the previous 2004 study (Bergek and Björklund, 2007), the barriers to dispersal corresponded to the deepest part in the lake. Thus, whatever the barriers are, physical or environmental, our results suggest that the barriers must be strong and stable over time.

Since we found a temporal genetic difference in every location, though not exceeding the spatial difference, this suggests that the fishes are moving around in the lake to a certain degree (i.e. within areas or patches). However, with the barriers being situated in the same areas in 2006 as 2 years previously, this indicates that they do not disperse and mix across long distances and that the spawning must be situated in different parts of the lake. Random drift of larvae that accidentally end up in the wrong location but within the same area would also explain our findings. An alternative explanation for the temporal difference within location may be the sampling of different cohorts among years. A between-cohort genetic difference is nonetheless only suspected in Location 5, where we found differences in length between the sampled fish. However, as length, our approximation of age, might not be rigorous enough, we do not exclude that there could be an effect of cohort on our results. To investigate this further, an exact method of age determination should be applied.

Alternatively, the presence of genetically differentiated populations might be explained by differences in allele frequencies among locations as a consequence of the sweepstake-chance matching in reproductive success (i.e. 'Hedgecock effect'). The sign of a 'Hedgecock effect' would be higher genetic heterogeneity over time in one location than seen spatially

among populations (Li and Hedgecock, 1998; Flowers *et al.*, 2002). In our study, the level of divergence was the same within as between years and stable over a sampling period of 2 years; hence it is unlikely that the spatial difference arising in the Lake is due to stochastic 'sweepstake effects'. The barriers to dispersal were unchanged and positioned in the same place in 2004 and 2006, further strengthening the conclusion of a true spatial structure. The perch populations are probably affected to some degree by the 'Hedgecock effect'. When the perch spawn, a female lays one long egg strand, after which several males have the possibility to fertilize the eggs (reviewed in Thorpe, 1977). If only a random sample of these survives to adulthood and the numbers of parents are small, there will be low N_e/N ratios and the populations will consist of genetically related individuals that swim in cohesive groups. Without demographic data, it is difficult to derive a precise estimate of N_e (Jorde and Ryman, 1995) but with the method used (Xu and Fu, 2004) we can at least obtain an informative estimate. Although one should be careful when making conclusions from this finding, our results indicate very small effective population sizes, supporting the sweepstake hypothesis where few individuals dominate the reproductive events. This could further explain the high relatedness values and elevated F_{IS}-values within the samples. In line with the 2004 study, our estimated relatedness values show that the fish in each location are related, but consist of several and not just one pure kin group (Bergek and Björklund, 2007); this would have given a 'false' signal of population differentiation. The high F_{IS}-values could also be due to technical artefacts (null alleles). Although we found evidence of null alleles at some loci, these were of a similar level as in the 2004 study and the corrected data set by MICRO-CHECKER did not alter the overall results. The reason for high F_{IS}-values is most likely in concordance with the scenario described above, with closely related individuals within each location ('family' Wahlund effect) giving rise to an overall homozygote deficiency.

Genetic divergence has been demonstrated previously in the Eurasian perch. At larger scales in Lake Constance, Gerlach *et al.* (2001) were able to detect two genetically distinct populations with an F_{ST}-value similar to those found at very small scales in Bergek and Björklund (2007) and in this study. Thus, population structure at both large and very small scales is likely in the species. In a recent paper, Behrmann-Godel and Gerlach (2008) presented data showing reduced fitness in crosses of the two genetically divergent populations in Lake Constance. This suggests that population divergence within lakes can be caused by intrinsic genetic incompatibility.

The results of this study reveal a stable spatial genetic differentiation at a very small geographical scale in Lake Erken with barriers to dispersal in the same locations, at least over a sampling period of 2 years. A temporal difference in each location was detected, suggesting that small populations/schools are moving around to a certain degree but not over the whole lake. This indicates the presence of different 'genetic patches' harbouring genetically differentiated groups of fish that are genetically more similar to each other within a patch than between patches. This is surprising given that in this species, the major gene flow might be mediated by planktonic larvae, something typically that results in low levels of genetic structure within biogeographical regions (Palumbi, 1992; Ward *et al.*, 1994; Bohonak *et al.*, 1999). This implies that dispersal of larvae in Lake Erken is limited and that separate perch breeding components persist over time. Furthermore, the Eurasian perch may also experience independent population dynamics, in agreement with Gold *et al.* (1993), Brown *et al.* (1996), and Ruzzante *et al.* (1997). This area of research is still in its infancy and more studies are needed to establish the extent and generality of the findings presented in this article.

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APPENDIX

Summary of the genetic data for six microsatellite loci from 2004 and 2006 (Locations 1, 3, 5, and 9). Number of individuals sampled (n), chi-square values and P-values calculated as deviations from Hardy-Weinberg equilibrium (HWE) for the observed and expected heterozygosity. The number of observed alleles (A_O) and gene diversity are shown for each location and locus.

Location	Locus	χ^2-value	P-value	A_O	Gene diversity
Location 1 (2004) ($n = 30$)	Svi4	—	—	—	—
	Svi7	0.30	N.S.	11	0.896
	Svi10	5.51	*	5	0.765
	Pfla1	1.98	N.S.	10	0.856
	Pfla6	2.11	N.S.	6	0.631
	Pfla9	4.56	*	9	0.747
Mean				8	0.779
Location 3 (2004) ($n = 30$)	Svi4	—	—	—	—
	Svi7	1.80	N.S.	11	0.903
	Svi10	3.21	N.S.	7	0.824
	Pfla1	0.86	N.S.	10	0.822
	Pfla6	0.81	N.S.	3	0.579
	Pfla9	2.38	N.S.	12	0.628
Mean				9	0.751
Location 5 (2004) ($n = 29$)	Svi4	1.30	N.S.	8	0.776
	Svi7	0.25	N.S.	9	0.875
	Svi10	1.20	N.S.	5	0.538
	Pfla1	0.00	N.S.	8	0.857
	Pfla6	3.20	N.S.	7	0.720
	Pfla9	2.03	N.S.	11	0.673
Mean				8	0.739
Location 9 (2004) ($n = 30$)	Svi4	0.01	N.S.	7	0.633
	Svi7	1.21	N.S.	11	0.881
	Svi10	0.01	N.S.	7	0.571
	Pfla1	3.41	N.S.	9	0.802
	Pfla6	2.45	N.S.	6	0.671
	Pfla9	1.88	N.S.	9	0.509
Mean				8	0.683

(*continued*)

APPENDIX—*Continued*

Location	Locus	χ^2-value	*P*-value	A_O	Gene diversity
Location 1 (2006) ($n = 45$)	Svi4	0.004	N.S.	8	0.584
	Svi7	5.40	*	11	0.884
	Svi10	0.21	N.S.	7	0.586
	Pfla1	2.02	N.S.	9	0.840
	Pfla6	5.38	*	5	0.718
	Pfla9	2.28	N.S.	10	0.681
Mean				8	0.714
Location 3 (2006) ($n = 44$)	Svi4	0.19	N.S.	7	0.763
	Svi7	3.36	N.S.	11	0.848
	Svi10	0.01	N.S.	5	0.513
	Pfla1	0.14	N.S.	9	0.824
	Pfla6	0.20	N.S.	6	0.658
	Pfla9	2.50	N.S.	12	0.718
Mean				8	0.721
Location 5 (2006) ($n = 45$)	Svi4	0.15	N.S.	7	0.555
	Svi7	3.36	N.S.	13	0.896
	Svi10	0.05	N.S.	6	0.609
	Pfla1	6.71	*	9	0.791
	Pfla6	2.23	N.S.	5	0.651
	Pfla9	0.74	N.S.	11	0.758
Mean				9	0.710
Location 9 (2006) ($n = 43$)	Svi4	0.55	N.S.	7	0.589
	Svi7	3.42	N.S.	13	0.898
	Svi10	0.26	N.S.	8	0.546
	Pfla1	8.05	*	8	0.832
	Pfla6	1.18	N.S.	5	0.646
	Pfla9	1.20	N.S.	14	0.715
Mean				9	0.704