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Genetic differentiation within and between populations of a hermaphroditic freshwater planarian

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Dispersal of individuals is an important factor that can influence genetic differentiation between populations. The hermaphroditic freshwater planarian *Schmidtea polychroa* inhabits shallow regions of lakes and streams, in which they appear to be continuously distributed. In the present study we used three highly polymorphic markers for analysing small-scale and large-scale genetic structure within one, and between four natural lake populations. Genetic differentiation could already be observed between samples collected at least 13 m apart, but not between neighbouring samples, and was most pronounced between samples from different lakes. Probably due to the high variance in F_{ST} values, a significant correlation between genetic differentiation and geographic distance could not be observed. These results show that individual dispersal of *S. polychroa* is limited, but that there is gene flow between subpopulations from the same lake. They further suggest that long-distance dispersal and gene flow between lakes, if present, is not a common process in *S. polychroa*.
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Keywords: *Schmidtea polychroa*; Plathelminthes; metapopulation structure; genetic diversity; dispersal; sexuality

Introduction

Genetic structure within, and gene flow between populations has been studied in many different species. The evolutionary significance of gene flow can be seen from different viewpoints: on the one hand, restricted gene flow may allow local adaptation and micro-evolutionary changes that increase fitness of local populations, while on the other, gene flow can be necessary to maintain genetic variation when population size is small (Slatkin, 1985, 1987). In finite populations, drift may lead to a loss of genetic variability in the neutral markers, usually applied in population studies, as well as allele composition at functional loci. For example, when heterozygosity yields a fitness advantage (Mitton, 1994), loss of genetic variation would lead to reduced heterozygosity and ultimately lower fitness. The effects of pronounced population structure (compared with a randomly mating population) have also been studied theoretically with regard to the evolution and maintenance of sex (Keeling and Rand, 1995; Peck *et al*, 1999). Although under certain ecological conditions, coexistence of sexuals and parthenogens is possible without spatial structuring of the population (Case and Taper, 1986), models and simulations that explicitly study the influence of population structuring revealed that sexuals are more likely to overcome the cost of sex when the population is highly structured (Keeling and Rand, 1995; Peck *et al*, 1999). The genetic structure of sexual populations is therefore of special interest when conspecific parthenogenetic populations are known.

The extent to which genetic variation and differentiation among populations can be observed, strongly depends on the markers used. In addition to the *F*-statistics commonly used to look for population differentiation, statistical procedures have been developed that either test the significance of the estimates for the *F*-statistics F_{IS} and F_{ST}, or use alternative methods to test for population differentiation (Rousset and Raymond, 1997; Luikard and England, 1999). Whereas allozyme markers often do not show enough polymorphism to resolve local differentiation, microsatellites can reveal high levels of genetic variability even in cases where allozymes were monomorphic. Microsatellite loci with 20 alleles or more in a normal population sample are not rare. In combination with computer programs that allow the calculation of the above-mentioned statistical tests on data sets with many populations, individuals and alleles per locus, they represent powerful tools for fine-scale population studies (overview in Luikard and England, 1999).

Natural populations of *Schmidtea polychroa*

Schmidtea polychroa is a non-selfing, hermaphroditic, freshwater planarian. It is present in many meso- and eutrophic freshwater lakes and streams in Europe. Individuals actively search for prey (snails, other freshwater invertebrates) (Calow *et al*, 1981), which implies that movement is an essential factor for foraging. Individuals may also move around in search of mates (see Greeff and Michiels, 1999). How far individuals move in nature is, however, unknown. Since long-distance dispersal of individuals and movements between isolated habitats may be rare in *S. polychroa*, genetic differentiation is expected between populations that are geographically isolated, for

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example different lakes. Within suitable habitats, individuals often appear to be continuously distributed, and it is unclear which spatial range a subpopulation comprises, and how big its effective population size is.

This study focuses on genetic variation within and among sexually reproducing populations of *S. polychroa*. In a study of sexual and parthenogenetic populations, allozymes proved to be sufficiently variable for determining clonal diversity of parthenogens and comparing sexual and parthenogenetic populations, but revealed only low within-population polymorphism, and even between geographically isolated populations, differentiation was quite weak (Pongratz *et al*, 1998). Therefore, three polymorphic microsatellite loci (Pongratz *et al*, 2001) were used for studying the genetic structure in a population where individuals appear to be homogeneously distributed along the shore, over a length of at least 1.5 km (Lago di Levico, Trentino). We wanted to estimate from which distance between two samples genetic differentiation can be observed, and whether a relationship between genetic and geographic distance exists (isolation-by-distance). Furthermore, we compared genetic differentiation within a lake, to that between lakes, where migration of individuals is supposedly rare or absent.

Methods

Field collection

The localities under study are on the northeastern shore of Lago di Levico (Trentino, Italy) where the shallow water zone in which most individuals are usually found, is narrow. Hence, sampling was basically one-dimensional. Adult individuals were collected according to a predefined sampling pattern (Figure 1). At three sites, one square meter (1×1 m^2) was chosen as smallest sampling unit, in order to test for fine-scale population differentiation in case of deviations from Hardy-Weinberg equilibrium. Three adjacent squares were later combined and referred to as subpopulations. Further subpopulation samples from different sites were collected from 4×1 m^2 rectangular areas. Pilot study samples were collected in 1997. In 1998, samples from subpopulations B, C, D, G, H and I were collected. In order to get a better resolution

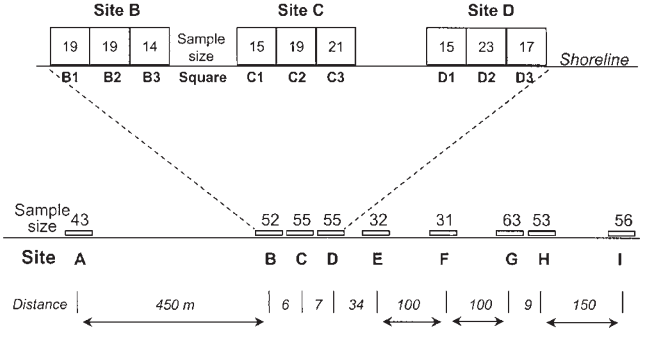

Figure 1 Sampling scheme along the eastern shore ('Strada di Pescatori') of Lago di Levico (Trentino, Italy). The smallest sampling unit was one square metre. For each site sample, individuals from three adjacent squares, or the respective area, were pooled. The fine-scale sampling from sites B, C and D was comprised of 3 $\times$ 3 square samples and allowed an exact test of population differentiation on a very small scale.

when testing for isolation-by-distance, samples from another three subpopulations (A, E, F) were collected in 1999 (Figure 1). Two samples of sexual individuals from Lago di Caldonazzo (2 km away from Lago di Levico, collected in 1998), one from the river Sarca (near Arco, Italy; 1998), and another one from Lago d'Iseo (Italy; 1998) were included in one analysis. Lago di Levico is a purely diploid, sexual population. For samples from populations in which polyploid parthenogens and diploid sexuals co-occur, karyology was used to distinguish both forms, as described by Beukeboom *et al* (1996). For analyses within one lake, samples from different sites are referred to as subpopulations. When comparing samples from different lakes, we shall refer to populations.

Microsatellite analysis

Tissue storage, DNA extraction and microsatellite analysis followed the protocols given in Pongratz *et al* (2001). For all samples, three loci were analysed (SpATT12, SpATT18, SpATT20).

Analysis of genetic variability and genetic structure

In order to describe genetic variability within subpopulations, standard indices of genetic diversity (number of alleles, observed and expected heterozygosity, gene diversity, F_{IS}) were estimated with the program FSTAT 2.7b (Goudet, 1995). *P*-values for F_{IS} within samples over all loci were calculated with FSTAT.

In order to detect on which level of geographic distance significant genetic differentiation can be found, differentiation between all pairs of subpopulations was measured with pairwise F_{ST} estimates. F_{ST} was preferred over alternative estimates that have been developed specifically for microsatellite data (Slatkin, 1995; Michalakis and Excoffier, 1996; Rousset, 1996), because the latter have a much higher variance than F_{ST} when based on few loci only (Gaggiotti *et al*, 1999). Pairwise F_{ST} and their respective *P*-values for significant differences from zero were calculated with ARLEQUIN 2.000 (Schneider *et al*, 2000). Isolation-by-distance was estimated following Rousset (1997), who proposed using pairwise F_{ST} values between populations for a correlation between $F_{ST}/(1 - F_{ST})$ and geographic distances in a one-dimensional array of populations. The matrix correlation was done with the program MANTEL (obtained from J Goudet, University of Lausanne).

GENEPOP 1.3d (Raymond and Rousset, 1995b) was used for exact tests of genotypic disequilibrium within subpopulations (Garnier-Gere and Dillmann, 1992) and pairwise population differentiation between subpopulations (Raymond and Rousset, 1995a; Goudet *et al*, 1996). The latter was also employed to statistically test for differentiation between adjacent sampling quadrants and subpopulations that were only few meters apart.

Results

Genetic diversity within subpopulations

Microsatellite polymorphism was high even within 1 m^2 quadrants which represented the smallest sampling unit. Within sites (3×1 m^2), nine to 22 alleles per locus were found. Gene diversity (D) was in the range of 0.85 to 0.94 with one exception, namely SpATT18 in sample M (Lago d'Iseo; D = 0.695) where only nine alleles were present,

three of which were extremely short and showed homozygote excess (Table 1). Most loci did not show significant within-sample deviations from Hardy-Weinberg equilibrium, and therefore F_{IS} values did not differ significantly from zero (Table 1). Allele ranges from different populations were overlapping to a large degree, with only few exceptions. For example, alleles with 24 to 28 repeat units at SpATT12 are absent or rare in the Lago di Levico subpopulation (A–I), but common in the Lago d'Iseo population (M). The exact test for genotypic disequilibrium did not yield significant results for any pair of loci except for sample sites E and F ($P < 0.05$ for all three pairs of loci).

Genetic population structure among sites within one lake

Differentiation between neighbouring quadrants within a site was not significant. Between some of the quadrants that were 20 m apart, significant differentiation was found. Differentiation between sites B and D was indicated by the F_{ST} that was significantly different from zero ($P < 0.0013$).

When all subpopulations from Lago di Levico were included, *F*-statistics revealed significant structuring of the population: an overall G-test (three loci) showed that F_{ST} differed significantly from zero ($P < 0.001$). The Mantel test for correlation of geographic distance and $F_{ST}/(1 - F_{ST})$ was not significant (observed matrix correlation = 0.2651; $P > 0.05$; Figures 2 and 3).

As expected, genetic structuring is also highly significant when samples from different lakes are included in the analysis. Pairwise F_{ST} values were clearly higher for samples from different lakes than for samples from the same lake (Table 2), and were in almost all cases significantly different from zero. We found a positive correlation between geographic distance between the populations and their genetic distance as estimated by $F_{ST}/(1 - F_{ST})$ (Mantel test; observed matrix correlation = 0.931; $P < 0.002$).

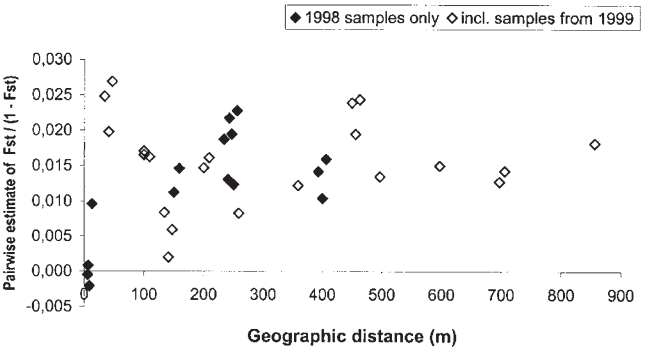

Figure 2 Relationship of genetic and geographic distance (metres). Genetic distance is represented by $F_{ST}/(1 - F_{ST})$ estimates. This plot shows that differentiation between populations, as measured by $F_{ST}/(1 - F_{ST})$, stays within the range from about 0.005 to 0.025 also beyond distances of 200 metres, and even within much shorter distances. It is not possible to resolve this, since the samples involved in the critical distance range (30–100 m) may not be appropriate with respect to sample size and genotypic disequilibrium (see text).

Discussion

Genetic diversity within natural populations

Microsatellites usually reveal high levels of allele size polymorphism, and proved to be very useful when variability at the allozyme level was not sufficient (Jarne and Lagoda, 1996). The high microsatellite variability present in the subpopulation samples from Lago di Levico confirmed previous results from other populations (including parthenogenetically reproducing ones) that revealed high microsatellite polymorphism within *S. polychroa* (Storhas, 2000). As expected, the level of polymorphism found with microsatellites was much higher than with allozymes that did not reveal any polymorphism in a small sample ($n = 20$) from Lago di Levico (site A).

Within all the samples collected in Lago di Levico, a

Table 1 Measures of genetic diversity at three microsatellite loci (a–c) in 13 samples from different populations

SpATT12	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*
n	43	52	55	55	30	30	63	53	56	38	52	89	24
H_{obs}	0.98	0.90	0.91	0.85	0.73	0.83	0.90	0.87	0.80	0.58	0.58	0.94	0.88
H_{exp}	0.91	0.87	0.90	0.88	0.87	0.91	0.92	0.93	0.93	0.88	0.85	0.84	0.84
F_{IS}	−0.065	−0.034	−0.002	0.034	0.175*	0.097	0.029	0.071	0.148*	0.356*	0.332*	−0.112	−0.018
A	18	19	20	17	14	18	20	20	21	16	14	20	10
SpATT18													
n	30	52	53	54	26	31	63	50	56	38	49	88	23
H_{obs}	0.80	0.92	0.92	0.87	0.96	0.90	0.87	0.96	0.89	0.89	0.98	0.89	0.35
H_{exp}	0.90	0.91	0.92	0.91	0.92	0.89	0.92	0.92	0.92	0.92	0.90	0.92	0.67
F_{IS}	0.130*	−0.004	0.004	0.058	−0.030	0.001	0.060	−0.034	0.036	0.042	−0.084	0.038	0.499*
A	16	19	21	21	17	14	20	19	21	17	15	22	9
SpATT20													
n	43	52	55	55	32	31	63	53	56	38	52	89	25
H_{obs}	0.81	0.85	0.91	0.89	0.97	0.81	0.71	0.77	0.89	1.00	0.69	0.90	0.80
H_{exp}	0.90	0.89	0.90	0.91	0.92	0.86	0.87	0.89	0.91	0.92	0.86	0.88	0.88
F_{IS}	0.107*	0.055	0.005	0.029	−0.039	0.077	0.191*	0.14*	0.031	−0.071	0.202*	−0.021	0.111
A	17	18	18	21	18	15	19	19	20	16	15	16	13

One column is used for each sample (n = sample size; H_{obs} = observed heterozygosity; H_{exp} = expected heterozygosity; F_{IS} = estimate of F_{IS} within samples, marked with an asterisk (*) if significantly different from zero; A = number of alleles sampled). Allele labels (first column) are expressed as the size in repeat numbers. A–I Lago di Levico, J, K = Lago di Caldonazzo, L = Sarca, M = Lago d'Iseo.

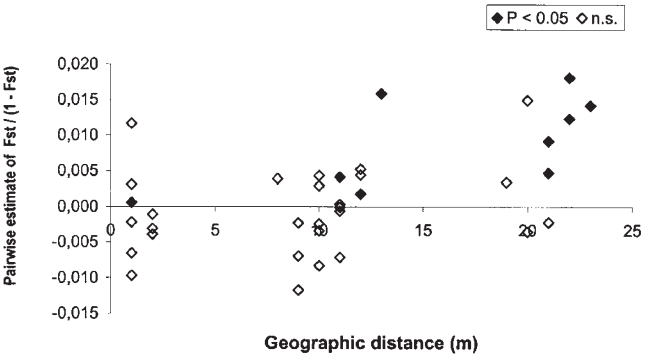

Figure 3 Fine-scale analysis for squares from sites B to D. The higher $F_{ST}/(1 - F_{ST})$ values are accompanied by significant tests for population differentiation. Adjacent squares and squares about 10 m apart show positive as well as negative $F_{ST}/(1 - F_{ST})$ values around zero, whereas more than half of the quadrants that are 20 m apart show significant differentiation.

total of 25 SpATT12, 28 SpATT18 and 24 SpATT20 alleles were found. An enormous amount of genetic variation is due to allelic variation within subpopulations, and even in 1 m^2 samples a considerable proportion of the alleles present in the pooled sample are represented.

The results of the tests for genotypic disequilibrium indicated significant linkage disequilibrium at two sites (E, F). This cannot be due to linkage of loci since it should be detectable in other samples as well, and mother-offspring comparisons also revealed that the three loci used here are not linked but segregate randomly (Pongratz *et al*, 2001).

Genetic differentiation between subpopulations

Genetic differentiation occurs when there is restricted gene flow between populations. The latter may be defined by single habitat patches that are separated by geographic or ecological factors that strongly restrict dispersal, for example, lakes or ponds in the case of freshwater organisms. However, each single patch may provide a continuous habitat that spans larger distances than can be covered by individual dispersal, and therefore genetic differentiation may also be observed within continuous populations, since matings between individuals will not be random with respect to distance between individuals (Wright, 1943). Several studies of different taxa reported stronger genetic differentiation of populations for species with limited dispersal abilities (references in Bohonak, 1999), and a negative relationship was found between dispersal ability and genetic differentiation in a meta-analysis of 333 studies (Bohonak, 1999). Fine-scale genetic differentiation has been studied in many plants (Linhart and Grant, 1996) and also animal species (eg, Johnson and Black, 1995; Chapuisat *et al*, 1997; Hitchings and Beebee, 1997; Johannesson and Tatarenkov, 1997). In this context, 'fine-scale', of course, strongly depends on the species' means of movement and dispersal.

The smallest sampling unit in this study was one square metre. Within these squares, differentiation was not expected. Local population or family structure within those squares could be indicated by deviations from Hardy-Weinberg-equilibrium, but only one out of 11 squares that were analysed with an exact test (unbiased estimates of Hardy-Weinberg exact *P*-values by the Markov chain method, GENEPOP) showed significant heterozygote deficiency ($P<0.01$). Two sets of individuals ($N_1 = 8$, $N_2 = 11$) collected from single stones (diameter of under-side surface about 10 cm) confirmed this assumption. They showed high levels of allelic variation that was not significantly different from that of the square where they were taken from (data not shown).

The results revealed significant structuring of samples taken from sites that were only about 13 m apart (Figure 3). This was surprising, since the results of sampling suggested that *S. polychroa* occurs along the whole stretch (about 20 m) that was covered by sites B, C and D, with no obvious dispersal barriers between sites.

Isolation-by-distance?

Differentiation between more distant sites was more pronounced, as indicated by higher F_{ST} values between pairs of subpopulations (Table 2; Figure 2), but we did not find a significant correlation between pairwise F_{ST} values and geographic distance between subpopulations, as predicted under the isolation-by-distance model. One reason for this may be that the variances for pairwise F_{ST} values calculated from three loci only are too high, and a correlation would require calculating the means over several measurements for each distance class. Another problem

Table 2 Pairwise F_{ST} values between samples (above diagonal) from different sites within Lago di Levico (A–I) and from other north Italian lakes (J, K = Lago di Caldonazzo, L = Sarca, M = Lago d'Iseo) and their respective *P*-values (below diagonal)

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*
A		0.0237	0.0194	0.0244	−0.0039	0.0108	0.0137	0.0146	0.0186	0.0376	0.0582	0.0564	0.1162
B	0.0001		−0.0005	0.0098	0.0109	−0.0005	0.0215	0.0224	0.0161	0.0397	0.0654	0.0669	0.1130
C	0.0001	0.5448		0.0011	0.0062	−0.0027	0.0142	0.0123	0.0105	0.0352	0.0613	0.0557	0.1038
D	0.0001	0.0013	0.3012		0.0092	0.0033	0.0214	0.0215	0.0145	0.0422	0.0615	0.0585	0.1105
E	0.8489	0.0013	0.0195	0.0050		0.0137	−0.0016	0.0043	−0.0037	0.0248	0.0503	0.0455	0.1065
F	0.0188	0.4985	0.7607	0.1630	0.0030		0.0111	0.0119	0.0015	0.0333	0.0624	0.0471	0.1071
G	0.0003	0.0001	0.0001	0.0001	0.7507	0.0026		−0.0026	0.0153	0.0411	0.0578	0.0501	0.1064
H	0.0010	0.0001	0.0001	0.0001	0.0912	0.0026	0.9326		0.0111	0.0381	0.0632	0.0500	0.1051
I	0.0001	0.0001	0.0003	0.0001	0.9395	0.3468	0.0001	0.0001		0.0248	0.0498	0.0484	0.0994
J	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001		0.0255	0.0560	0.0998
K	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001		0.0665	0.1121
L	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001		0.0932
M	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	

could be that two of the three samples from 1999 that were added to get intermediate (30–100 m) as well as longer (>500 m) geographic distances, had clearly smaller sample sizes than the rest. These also included unusually high levels of missing data at SpATT18, and showed linkage disequilibrium at all pairs of loci. This may have resulted in, for example, the high values between sites E and sites B, C and D (Table 2; Figure 2). Furthermore, finding isolation-by-distance requires sampling at the appropriate spatial scale, and therefore, the pattern of isolation-by-distance may be not observed within the chosen range of distance values (Rousset, 1997). Due to sampling restrictions that are necessary in experimental studies, the scale at which samples were taken may not be appropriate for determining the relationship between genetic and geographic distance, although it may be possible to fit the data to a linear model and calculate dispersal parameters as suggested by Rousset (1997).

An implicit assumption of the isolation-by-distance analysis was that the areas between sites are equally suitable for individuals to migrate. Although there were no obvious obstacles within the range of the study sites in Lago di Levico, it cannot be excluded that such obstacles existed. When movement between subpopulations is common between some sites but restricted between others, even if they are close together, genetic differentiation will be more pronounced between the latter. Similarly, ecological heterogeneity of the shoreline could also be responsible for the observed differences in the degree of differentiation between sites, irrespective of their geographic distance. Several studies in plants have shown that environmental and genetic heterogeneity are often associated (Linhart and Grant, 1996). In nature, both habitat and geographic distance can influence differentiation between subpopulations to varying degrees, as has been shown in the snail *Littorina saxitalis* by Johannesson and Tatarenkov (1997). For *S. polychroa*, ecological heterogeneity along the shoreline may, for example, exist with respect to food supply, vegetation, substrates available for cocoon attachment, and presence of competitors and predators. In the present study, reed vegetation along the shore altered with open stretches without vegetation, introducing environmental heterogeneity to a certain extent. *S. polychroa* can be found in both environments. Sampling sites included both open and reed-overgrown stretches but was concentrated on open stretches. Therefore, it is not possible to analyse effects of such potential ecological differences with the available data regarding habitat-specific variation. However, the data suggest that dispersal is not affected by reed and open stretches, as there was no differentiation observed between sites B and C, which are separated by a 3 m zone of reeds. Ecological heterogeneity along the shoreline may also be introduced by small tributary creeks, that can alter the quality, temperature, or nutrient-richness of the water. Although species abundance and distribution may be strongly affected by such factors, it is not clear whether it would influence the genetic structure of subpopulations as well.

Finally, it should be pointed out that the results of our study may mix temporal and spatial effects on the population structure since the samples from Lago di Levice were collected in different years. If the temporal structure was stronger than the spatial genetic structure (Viard *et al*, 1997), genetic differentiation that occurs between seasons or years could erroneously be attributed to spatial differences between the sites.

Differentiation between lakes

F_{ST} estimates obtained from the analysis of populations from different lakes are higher than the ones observed within one lake. Between populations from different lakes, migration may be absent or negligible. If individuals moved between Lago di Caldonazzo and Lago di Levico regularly, for example by passive dispersal via waterfowl, polyploid parthenogenetic individuals that are abundant in the former, should also be observed in the latter, but there is no indication for polyploids in Lago di Levico. Therefore, Lago di Caldonazzo populations may serve as reference samples. F_{ST} values between more distant populations are higher due to substantial differences in allele frequencies. The allele size ranges of the three loci, however, overlapped almost completely in all populations, with few exceptions that mainly concerned rare alleles, and the population M from Lago d'Iseo (SpATT18, data not shown). The correlation between genetic and geographic distance was significant when samples from different lakes were analysed. This could be interpreted as isolation-by-distance, implying that there is migration between the lakes. It is, however, also possible that the closer genetic relationship between geographically close populations is due to more recent common ancenstry, and genetic differentiation is not yet as pronounced as between populations that are further apart. With regard to our sample areas in northern Italy, the latter appears more likely since the respective areas have only been recolonised after the last glaciation. Evidence from a mitochondrial DNA sequencing study (Pongratz, 2002) also strongly indicates that colonisation history has had a strong influence on the genetic structure of these populations.

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